Pharmacological characterization of human neutrophil prostanoid EP receptors: modulation of neutrophil function

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PHARMACOLOGICAL CHARACTERIZATION OF HUMAN NEUTROPHIL PROSTANOID EP RECEPTORS: MODULATION OF NEUTROPHIL FUNCTION

submitted by

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ABSTRACT

The potency order of prostanoid EP receptor agonists which inhibited fMLP-stimulated superoxide generation and stimulated cAMP generation by human neutrophils were found to be consistent with the EP_2 receptor subtype. However, evaluation of prostanoid receptor antagonists showed that AH 6809 (prostanoid DP and EP_1 receptor antagonist), specifically and selectively antagonised PGE_2-mediated inhibition of superoxide generation. Furthermore, PGE_2-stimulated cAMP elevation in human neutrophils was also antagonised by AH 6809 with a similar potency. The results suggested that contrary to the existing classification of prostanoid EP receptors, AH 6809 antagonises an EP_2-mediated response. However, it is unclear whether this neutrophil prostanoid EP receptor subtype may represent a novel subtype or a human homologue of the classical EP_2 receptor. Studies investigating the presence of 'EP_n' receptors on rabbit peripheral blood neutrophils were inconclusive, therefore the species specificity and distribution of the 'EP_n' receptor are unclear.

The signal transduction pathway utilized by this neutrophil prostanoid EP receptor, termed 'EP_n', appeared to be via stimulation of adenylate cyclase and cAMP elevation. In addition, cAMP increases in the neutrophil may modulate neutrophil function by attenuating influx-dependent increases in $[Ca^{2+}]_i$ associated with neutrophil activation, but had no effect on the release of Ca^{2+} ions from intracellular stores.

'EP_n' receptors mediating cAMP elevation were also present on human monocytes and the human promyelocytic cell line (HL-60) as defined by AH 6809 antagonism of PGE_2. However, AH23848B (EP_4 receptor antagonist) non-competitively antagonised the PGE_2-mediated cAMP increase, suggesting that human monocytes may also express prostanoid EP_4 receptors.

Therefore, inhibitory prostanoid 'EP_n' receptors are present on human neutrophils and monocytes and may represent a novel therapeutic target for the development of anti-inflammatory drugs.

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ABBREVIATIONS

α	effect of an agonist normalised with respect to the maximum tissue response
ARDS	adult respiratory distress syndrome
ATP	adenosine trisphosphate
AUC	area under curve
BSA	bovine serum albumin
C5a	complement fragment 5a
$[Ca^{2+}]_i$	cytosolic free calcium ion concentration
cAMP	cyclic 3', 5' adenosine monophosphate
CGD	chronic granulomatous disease
CO_2	carbon dioxide
COX	cyclooxygenase
DAG	sn-1,2-diacylglycerol
E/[A] curve	agonist concentration-effect curve
EDTA	di-sodium ethylene-diamine-tetraacetic acid
EGTA	ethylene glycol-bis[β-aminoethyl ether] N,N,N',N'-tetraacetic acid
EP	E-type prostaglandin receptor
FCS	foetal calf serum

fMLP	formyl-methionyl-leucyl-phenylalanine
Fura-2 am	fura-2 acetoxymethyl ester
GMCSF	granulocyte-macrophage colony-stimulating factor
GTP	guanine trisphosphate
HBSS	Hanks buffered salt solution
H_2O_2	hydrogen peroxide
HSA	human serum albumin
IL-1	interleukin-1
IL-8	interleukin-8
IP_3	inositol (1,4,5)-trisphosphate
LPS	lipopolysaccharide
LT	leukotriene
NO	nitric oxide
O_2^-	superoxide anions
OZ	opsonised zymosan
$p[A_{50}]$	negative $\log_{10}$ molar concentration of agent producing 50% of its own maximum response
PDEI	phosphodiesterase inhibitor
$p[EC_{50}]$	negative $\log_{10}$ molar concentration of agent producing 50% of maximum response

$p[IC_{50}]$	negative $\log_{10}$ molar concentration of agent producing 50% inhibition of a given response
PAF	platelet activating factor
PBS	phosphate buffered saline
PG	prostaglandin
PIP_2	phoshatidylinositol (4,5)-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PPP	platelet poor plasma
PRP	platelet rich plasma
RANTES	regulated on activation, normal T-cell expressed and secreted
RIA	radioimmunoassay
RMCE	receptor mediated calcium entry
ROC	receptor operated calcium channel
SMOC	second messenger operated calcium channel
SPA	scintillation proximity assay
TNF_α	tumor necrosis factor-α
TX	thromboxane
VOC	voltage operated calcium channel

CHAPTER 1

GENERAL INTRODUCTION

1.1 Leukocytes in host defence

Immunity is the body's defence system, its protective mechanisms recognise and eliminate or neutralise foreign or abnormal materials. These immune responses are provoked by microbial infection and damaged or abnormal cells. The cells of the immune system are leukocytes normally found in the circulation, but migrate to all parts of the body to perform this host defence (Brent, 1992).

There are two main types of immune response, specific and non-specific. The former requires prior exposure to specific foreign substances (antigens), and recognition of antigen by leukocytes of the lymphocytic lineage on subsequent exposure. Non-specific immune responses, commonly known as the inflammatory response, are mounted against all foreign materials and do not require pre-exposure or recognition of antigenic determinants (Van Arsdel, 1992).

1.1.1 Leukocytes and inflammation

Inflammation is triggered during the initial exposure to a foreign substance (before specific immunity is activated) and in response to any tissue injury. The purpose of the inflammatory response is to bring leukocytes to the site of injury to destroy or inactivate the foreign material and prepare the way for tissue repair (Van Arsdel, 1992). Neutrophils are the earliest leukocytes to arrive at the scene and provide the first line of defence. When the cause of the insult is successfully eliminated, the acute inflammation remains localised and quickly resolves. If the material is not eliminated, the inflammation persists and leads to the recruitment of monocytes and lymphocytes into the inflamed area. A granuloma is subsequently formed to isolate the inflammogen, but if it is not completely effective, develops into a chronically inflamed lesion. Chronic inflammatory conditions such as rheumatoid arthritis (Van Arsdel, 1992), asthma (Kay, 1986), inflammatory bowel disease (Yamada & Grisham, 1991) and psoriasis (Christophers *et al.*, 1982) are characterized by periods of regression and repair, punctuated by further acute inflammatory episodes involving leukocyte (neutrophil, monocyte and lymphocyte)

infiltration and activation.

The leukocytes which perform host defence are of an aggressive destructive nature which need to be highly regulated to protect the host, without inflicting tissue damage and inflammation on the host. Dysfunction of these regulatory processes leads the inappropriate and excessive leukocyte activation, as occurs in acute inflammation (adult respiratory distress syndrome, ARDS, Windsor *et al.*, 1993), chronic inflammation (rheumatoid arthritis, Van Arsdel, 1992) and autoimmune diseases (systemic lupus erythematous disease, Isenberg, 1992). Amongst the most potentially destructive leukocytes are the 'professional phagocytes' such as monocytes, eosinophils and neutrophils.

1.1.2 The role of the neutrophil in inflammation

The neutrophil is an end-stage cell incapable of division with a short life-span (half-life of approximately 12 hours in the circulation, McAfee *et al.*, 1976), and limited protein synthetic capacity. Although the neutrophil is generally associated with acute inflammation, it has a surprisingly wide range of responses which can cause considerable tissue damage. In addition, neutrophil function is regulated by a wide range of endogenous and exogenous agents.

Circulating neutrophils normally make only transient contact with endothelial cells lining the post capillary venules, by rolling along the luminal surface of the endothelial cells, after which, neutrophils detach themselves and return to the general blood flow. Neutrophil rolling involves labile interactions between complementary selectin adhesion molecules expressed on the plasma membranes of the neutrophils and endothelial cells (Lawrence & Springer, 1991). However at sites of inflammation, neutrophils migrate out of the circulation into the extravascular tissue in response to inflammatory agents released as a result of the trauma, to eliminate the inflammogen. The sequence of events in neutrophil activation in inflammation is illustrated in Fig 1.1 and described below.

The process of neutrophil extravascular migration is believed to consist of 3 sequential steps. The first step is the initial tethering of the neutrophils to the endothelial cells in the vicinity of the inflammatory insult, by interacting with different selectin adhesion molecules, such as E-selectin. Endothelial cells do not constitutively express E-selectin molecules and requires *de novo* protein synthesis. Inflammatory cytokines such as tumor necrosis factor-α (TNF_α) and interleukin-1 (IL-1), and lipopolysaccharide (LPS), a constituent of bacterial cell walls, however stimulate E-selectin expression by endothelial cells (Lawrence & Springer, 1991). The selectin-mediated tethering brings neutrophils into the proximity of chemoattractants (discussed in the next section) released from or displayed by endothelial cells for the second step of the migration process-neutrophil activation. These chemoattractants stimulate neutrophils to activate their surface expression of MAC-1 (CD11b/CD18) integrin molecules, the complimentary ligand for the ICAM-1 integrin adhesion molecule expressed by endothelial cells which mediates firm leukocyte-endothelial cell adhesion (Larson & Springer, 1990). IL-1 and TNF_α also upregulate the expression of ICAM-1 (Lawrence & Springer, 1991), which facilitates the integrin mediated adhesion which stops the neutrophils rolling. Chemoattractant-stimulated neutrophil integrin activation is due to a conformational change of the expressed integrin receptor and is not dependent on upregulation of additional integrins. Neutrophil activation by chemoattractants causes a simultaneous shedding of selectin adhesion molecules (Kishimoto *et al.*, 1989), and is required for the next step of neutrophil migration. However, integrin receptor interactions between neutrophils and endothelial cells are required for the final step, neutrophil diapedesis, the process of transmigration from the intravascular lumen via the inter-endothelial junctions into the extravascular tissue to reach the site of tissue damage (Arfors *et al.*, 1987).

After passing through the endothelial barrier, neutrophils migrate towards the inflammatory stimulus, down a concentration gradient, by a process called chemotaxis. On encountering the cause of the inflammation, neutrophils will attempt to destroy the

foreign matter by releasing degradative enzymes such as β-glucuronidase from their azurophilic granules and the generation of toxic reactive oxygen metabolites (ROM) by activation of NADPH-oxidase (commonly known as the respiratory burst). Particulate stimuli such as bacteria and tissue debris are engulfed by neutrophils into discrete intracellular pockets called phagosomes. The contents are destroyed by fusion of granules with the phagosome and the emptying of the enzymes into the pocket, and by the release of ROM into the lumen of the phagosome by NADPH-oxidase located in the phagosome membrane. Neutrophils also generate proinflammatory factors which can recruit, prime and activate other leukocytes, such as leukotriene B_4 (LTB_4, Ford-Hutchinson *et al.*, 1980) and platelet activating factor (PAF, Lotner *et al.*, 1980) by activating phospholipase A_2 (PLA_2). Neutrophils can synthesize proteins, such as interleukin-8 (IL-8) on stimulation by cytokines (Strieter *et al.*, 1990) and during phagocytosis (Bazzoni *et al.*, 1991). 'Frustrated phagocytosis' by neutrophils may contribute to the tissue damage, when neutrophils are unable to completely engulf particulate stimuli and tissue debris. ROM and proteolytic enzymes are then released into milieu and damage host tissue rather than the contents of fused phagosomes.

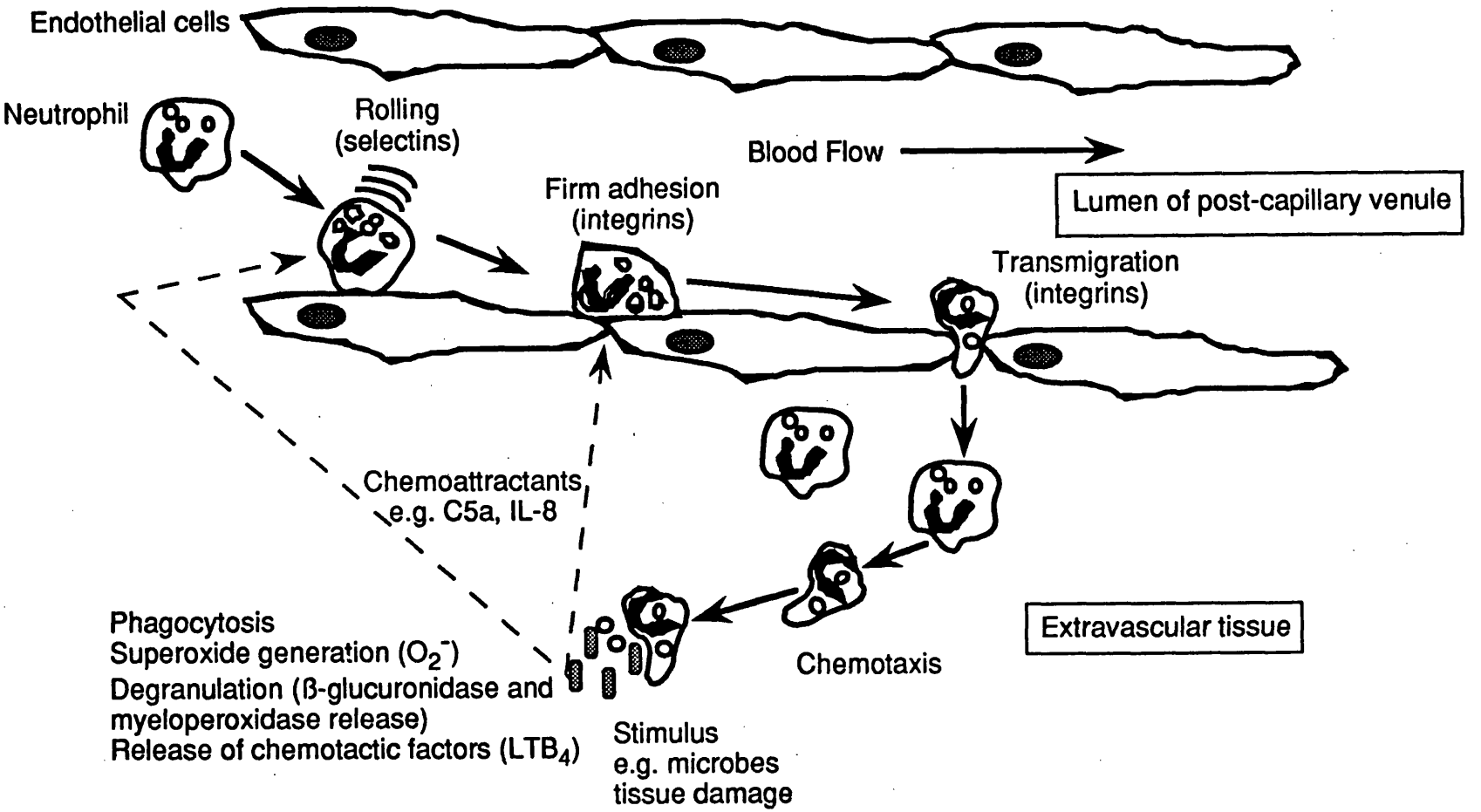


Fig 1.1 Illustration of the sequence of events involved in neutrophil activation at sites of acute inflammation

1.1.3 Effect of inflammatory mediators on neutrophil function

Neutrophils can be activated by a range of endogenous stimuli in addition to bacterial-derived agents such as the tripeptide formyl-methionyl-leucyl-phenylalanine (fMLP). Endogenous inflammatory mediators such as phospholipid derived LTB_4 and PAF released from neutrophils (Wymann *et al.*, 1987), and products from the activation of the complement cascade, the peptide complement factor C5a (Hugli & Chenoweth, 1981). More recently, a group of chemotactic cytokines (chemokines) have been discovered (reviewed by Oppenheim *et al.*, 1991), the first one described was the neutrophil selective chemokine interleukin-8 (IL-8). Subsequently more neutrophil selective chemokines have been discovered such as ENA-78 (epithelial neutrophil activating peptide 78) and neutrophil-activating peptide-2 (NAP-2). Another family of monocyte and eosinophil selective chemokines has also been discovered, whose members include monocyte chemotactic peptide 1,2 and 3 (MCP-1, -2 and -3) and RANTES (reviewed by Oppenheim *et al.*, 1991).

Neutrophil activation stimulated by these chemoattractants, C5a, LTB_4, IL-8 and fMLP, is mediated by specific receptors for these stimuli on the plasma membrane. The receptors are the classical rhodopsin type of G protein coupled receptors, with 7-transmembrane spanning domains (Holmes *et al.*, 1991). Activation of neutrophil chemoattractant receptors stimulates increases in intracellular calcium, chemotaxis, degranulation and the respiratory burst (Snyderman & Uhing, 1992, and Baggiolini & Kernen, 1992).

IL-8 may be the most important endogenous neutrophil chemoattractant in inflammation, as IL-8 is generated by a wide variety of cell types. Leukocytes such as monocytes, lymphocytes and neutrophils synthesise IL-8 (Gregory *et al.*, 1988, Walz *et al.*, 1987, Strieter *et al.*, 1990, respectively), as do non-immune cells, including fibroblasts (Van Damme *et al.*, 1989), synovial cells (Watson *et al.*, 1988a,b), chondrocytes (Lotz *et al.*, 1992) and endothelial cells (Schröder & Christophers, 1989). In addition, IL-8 may play

an important role in neutrophil emigration, as endothelial cells present IL-8 molecules on their luminal surface (Rot, 1992), which would activate rolling or adherent neutrophils. Studies have shown that endogenous IL-8 may regulate neutrophil migration by inducing selectin shedding to facilitate spreading and transmigration (Huber *et al.*, 1991), and anti-IL-8 monoclonal antibodies inhibit neutrophil emigration in the rabbit (Sekido *et al.*, 1993).

Cytokines other than the chemokines, for example, IL-1, TNF_α and granulocyte-macrophage colony stimulating factor (GMCSF) may also regulate neutrophil activation, but they differ from chemokines in several ways. The receptors for interleukin 1 (IL-1), (TNF_α) and granulocyte-macrophage colony stimulating factor (GMCSF) are type I membrane glycoproteins containing a single transmembrane domain (Taniguchi, 1995). However, IL-1 does not appear to have a direct effect on the neutrophil. IL-1 stimulated neutrophil activation *in vivo* may be mediated via induction of IL-8 generation (Watson *et al.*, 1988a). In contrast, TNF_α and granulocyte-macrophage colony stimulating factor (GMCSF) directly influence neutrophil activation. They are unable to elicit a response from neutrophils *in vitro* themselves, but prime neutrophils to be more readily activated and more responsive to subsequent stimulation by the direct neutrophil stimuli described above (Khwaja *et al.*, 1992). The exact mechanism involved in the priming of neutrophils however is not clear, although a role for phospholipase D and phosphatidic acid has been suggested (Bauldry *et al.*, 1991), and may be involve the generation of PAF (DeNichilo *et al.*, 1991, Stewart *et al.*, 1991). In addition, the cytokines, TNF_α and GMCSF, and the bacterial cell wall derived lipopolysaccharide (LPS), are able to stimulate hydrogen peroxide generation by adherent (to endothelial cells and basement membrane proteins such as fibrinogen), but not suspended neutrophils (Nathan, 1987). The ability of TNF_α and GMCSF to stimulate neutrophils directly therefore, may depend on interactions between their receptors, the cytoskeleton and adhesion molecules on adhesion to endothelial cells and extracellular proteins (Nathan, 1987).

1.1.4 Modulators of neutrophil activation

Modulation of neutrophil activation would be an attractive therapeutic target as a treatment of inflammatory conditions where there is excessive or inappropriate neutrophil recruitment and activation. Several approaches are available to modulate leukocyte activation which are applicable to neutrophils:-

a) Preventing the activation of leukocytes with antagonists of specific leukocyte stimuli, for example IL-8 receptor antagonists

b) Modulating signal transduction pathways regulating leukocyte function by inhibiting stimulatory signal transduction pathways or stimulating/enhancing inhibitory intracellular second messengers such as cAMP

1.1.4.1 Inhibition of neutrophil activation with antagonists of specific neutrophil stimuli

There are a large number of exogenous and endogenous stimuli which are able activate or prime neutrophils (see section 1.1.3). However, the type of stimuli present at an inflammatory site depends on the cause of the inflammation, for instance, bacterial infection or tissue damage, and the location of the injury, such as the skin or the lung. Any combination of inflammatory mediators could be present. Therefore, inhibiting neutrophil activation by antagonising single specific stimuli may only be effective in a limited range of inflammatory responses. The stimuli involved would not be the same in every disease and may not be known in some inflammatory conditions, hence, antagonists of specific neutrophil stimuli may not be therapeutically useful.

1.1.4.2 Inhibition of neutrophil activation by modulating intracellular transduction pathways

The neutrophil chemoattractants, such as fMLP and C5a, stimulate G protein coupled receptors to activating the classical activation pathway involving phospholipase C

activation. This pathway activates protein kinase C and elevates intracellular calcium levels (Cockcroft & Gomperts, 1985). Activation of these signal transduction molecules leads to activation of the neutrophil stimulating degranulation and the respiratory burst.

There are also intracellular signal transducer molecules which inhibit leukocyte activation. The most ubiquitous and most well known inhibitory second messenger is cyclic 3',5'-adenosine monophosphate (cAMP). Adenylate cyclase catalyses the formation of cAMP from adenosine trisphosphate (ATP), whilst cAMP is inactivated by phosphodiesterases by conversion to 5' adenosine monophosphate. Cell-permeable analogues of cAMP such as dibutyryl cAMP, or agents which elevate endogenous cAMP levels either by activating adenylate cyclase (forskolin) or inhibiting phosphodiesterase (isobutylmethyl xanthine, IBMX), inhibit neutrophil activation (Lad *et al.*, 1985, Nourshargh & Hoult, 1986, Lad *et al.*, 1985). Therefore, agents which elevate cAMP may be potentially anti-inflammatory.

1.1.4.3 Inhibition of neutrophil activation by phosphodiesterase inhibitors

There at least 5 subtypes of phosphodiesterase for which cAMP is a substrate, and isoforms exist within individual PDE subtypes (reviewed by Beavo, 1990), but the type IV cAMP-specific PDE is found in most leukocytes. Inhibitors of the type IV isozyme suppress leukocyte activation *in vitro* and cause relaxation of smooth muscle, and are therefore potentially anti-inflammatory and bronchodilatory (Torphy & Undem, 1991). Several selective type IV PDE inhibitors have been developed for the treatment of asthma but exhibited antidepressant activity in clinical trials (Watchel, 1983). These side effects may be attributable to the lack of selectivity of the inhibitors for the different isozymes of type IV PDEs.

1.1.4.4 Inhibition of neutrophil activation by stimulators of adenylate cyclase

There are at least 8 different isoforms of the mammalian adenylate cyclase (Kruprinski *et al.*, 1992), but the isoform present in human leukocytes has not been identified.

Forskolin is a direct-acting non-selective stimulator of adenylate cyclase (De Souza *et al.*, 1983), and also lacks potency. Therefore development of a selective activator of the neutrophil adenylate cyclase may be difficult to achieve. However, neutrophils and other leukocytes express plasma membrane receptors for substances which suppress their activation, for example, adenosine (Cronstein *et al.*, 1983), adrenergic agents (Lad *et al.*, 1985), histamine (Burde *et al.*, 1990) and prostaglandins (Bourne *et al.*, 1971). These inhibitory mediators may act as endogenous physiological modulators of leukocyte activation. The development of selective agonists for these inhibitory receptors may represent another approach for the development of novel anti-inflammatory agents. These inhibitors may be more useful as anti-inflammatory agents as they are not as specific as the antagonists of leukocyte stimuli. They may encounter the problems of selectivity associated with modulators which act intracellularly, such as phosphodiestase inhibitors, but these may be more easily identified and overcome. The pharmacological characterization of receptors on the plasma membrane is more readily performed in intact cells, as opposed to intracellular targets which would require extensive biochemical isolation and purification before characterization could be performed.

The receptor-orientated agonistic approach is perhaps the more amenable to pharmacological investigation and intervention, and has been undertaken in the studies presented in this thesis on the human neutrophil. Although the neutrophil may not be most important cell in inflammation, it is perhaps the most suitable leukocyte for such a study. Neutrophils are the most numerous of the leukocytes in the circulation, and their responses *in vitro* are comparatively simple, have a rapid onset and short duration compared to lymphocytes, monocytes and eosinophils. The majority of the pharmacological analysis performed in the present study will be performed using human neutrophils but will be extended to other leukocytes.

One of the best known inhibitors of leukocyte activation are the E-type prostaglandins, as initially reported by Gordon *et al.* (1976), who demonstrated inhibition of T-lymphocyte activation by PGE_2 *in vitro*. Subsequently, the immunomodulatory effects of PGE_2 have

been observed in lymphocytes, monocytes and neutrophils *in vitro* and *in vivo* (reviewed by Kunkel & Chensue, 1984). However contradictory reports of proinflammatory effects of prostaglandins have also been described, but it is unclear whether these are attributable to different prostanoid receptor subtypes (reviewed by Goodwin, 1991). In addition, the classification of prostanoid receptors has been conducted in other tissues and subtypes of prostanoid receptors have been identified (Gardiner & Collier, 1980 and Kennedy *et al.*, 1982). In spite of this, leukocyte inhibitory prostanoid receptors have not been pharmacologically characterized prior to the initiation of this study.

1.2 Prostaglandins and their receptors

1.2.1 Historical and general background

The term "eicosanoids" encompasses the unsaturated lipids derived from C_{20} polyunsaturated fatty acid precursors, the most common being arachidonic acid ($C_{20:4\ n-6}$). They can divided into two groups; those formed via the cyclo-oxygenase pathway, namely the prostanoids (prostaglandins and thromboxanes) and the products of the lipoxygenase metabolic route (leukotrienes, lipoxins and hydroxy-fatty acids).

Prostaglandins are oxygenated polyunsaturated 20-carbon fatty acids containing a cyclopentane ring (a characteristic of the theoretical parent structure prostanoic acid). Thromboxanes, on the other hand, contain an oxane ring, and may be regarded as derivatives of thrombanoic acid.

The biological activity of prostaglandins was first discovered in the 1930s as a factor found in human semen and sheep vesicular gland which stimulated smooth muscle and lowered blood pressure (Goldblatt, 1933, and Von Euler, 1934). Von Euler (1936) characterised this factor as a hydroxylated unsaturated fatty acid, and the name prostaglandin was conferred on the assumption that it originated from the prostate gland.

Prostaglandins (PGs) are not synthesised and stored like most hormones, but are synthesised de novo by almost all mammalian cells and act locally as autocoids. They are generated in response to a wide range of stimuli; physiological, such as hormone receptor activation, and pathological, such as mechanical trauma. There are three series of naturally occurring PGs, 1-,2- and 3- according to the fatty acid precursor (constituents of membrane phospholipids) they are derived from. The numerical subscript indicates the number of double bonds in the alkyl chain. The 1-series is derived from eicosatrienoic acid ($C_{20:3\ n-6}$), the 2-series from eicosatetraenoic acid ($C_{20:4\ n-6}$ more commonly known as arachidonic acid) and the 3-series from eicosapentaenoic acid ($C_{20:5\ n-3}$). The PGs of 2-series i.e. derived from arachidonic acid are predominant in mammalian tissue and their

synthetic route is shown in Fig 1.2.

1.2.2 Classification of prostanoid receptors

The prostanoids are ubiquitously present in the mammalian body, capable of exerting a wide spectrum of biological actions on different tissues, organs and cells and proposed to have pathophysiological roles such as inflammatory and peripheral vascular diseases. Prostanoids have a multiplicity of target tissues and are able to elicit a wide range of responses, some of which are opposing in the same tissue such as contraction and relaxation of smooth muscle but at different concentrations (Gardiner & Collier, 1980). These observations have hampered the progress of prostaglandin research investigating the involvement of individual prostanoids and their receptors in inflammatory processes and pathology of diseases such as ARDS, asthma, inflammatory bowel disease, psoriasis and arthritis.

Hence, the classification of prostanoid receptors in general was imperative to determine which responses were attributable to which receptors. Such a classification would also lead to the discovery of selective agonists and antagonists which would reciprocally aid receptor classification, and provide possible therapeutic starting points for the discovery of potential drugs to treat these diseases.

Two different approaches were undertaken to form a preliminary classification of prostanoid receptors. Coleman *et al.* (1984) conducted a systematic search of a wide range of tissues to identify those which they believed had a homogeneous receptor population. This was achieved by applying two criteria; the tissues should respond with either a relaxation or contraction (not both) to each prostanoid agonist, and secondly in each tissue, one naturally occurring prostanoid was considerably more potent. These preliminary studies identified specific receptors for PGE_2, $PGF_{2\alpha}$ and TXA_2. The incorporation of data from studies on platelets (human and guinea-pig) led to the proposal of the existence of five distinct receptor types for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and

TXA_2. The nomenclature proposed for this classification was that prostanoid receptors be termed P-receptors and the prostanoid which was most potent at the receptor indicated by a prefix, for instance, DP, EP, FP, IP and TP receptors for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 respectively. Subdivisions of the receptors were indicated by numerical subscript, for example, EP_1, EP_2, EP_3, etc.

The other approach (Gardiner & Collier, 1980) used a limited number of tissues (guinea-pig trachea and human bronchial muscle). Responses of these respiratory tissues to PGE_1, PGE_2, $PGF_{2\alpha}$, $PGF_{2\beta}$, PGI_2 and 3 synthetic prostanoid agonists (TR4161, TR4367 and TR4752) were classified as contractile/stimulant (χ), relaxant/inhibitory (ψ) or "irritant" (ω) (they induced cough in cats). These receptors were later subdivided (Gardiner, 1986) on the basis of agonist potency orders in a wider range of tissues (respiratory and non-respiratory), and the activity of the ligands; U46619 (thromboxane mimetic) and SC 19220 (EP receptor antagonist). The contractile prostanoid receptors were subdivided into χ_1 receptors (agonist U46619), χ_2 receptors (agonist $PGF_{2\alpha}/PGD_2$) and χ_3 (agonist PGE_2/antagonist SC 19220). The relaxant receptor (ψ) was also subdivided as agonist potency orders for PGE_1, PGE_2, PGI_2 and butaprost (EP receptor agonist, TR4979) were different for inhibition of human platelet aggregation and vasodilation (Copas *et al.*, 1981).

The activity of a prostanoid in a tissue does not necessarily indicate that its specific receptor is present in the tissue. Only the use of selective antagonists can determine this, as shown by AH19437 (TP receptor antagonist) antagonism of U46619 at TP receptors and the weaker effects of other prostaglandins at this receptor. This illustrates the promiscuity of prostanoids and their receptors and why the Coleman *et al.* classification was more easily understood and universally adopted. The Gardiner & Collier approach used a limited number of tissues which clearly contained

heterogeneous prostanoid receptor populations, and the classification was not easily transferable from respiratory tissues to other organs.

Selective agonists and antagonists have however been identified using these criteria, such as the EP_1/EP_3 selective agonist sulprostone and AY23626 (Coleman *et al.*, 1987). Their use has clarified the preliminary classifications of both Coleman *et al.* and Gardiner which in turn led to the development of more selective ligands. This self-perpetuating process has evolved into the classification which is currently recognised today as being as unambiguous and pharmacologically correct as possible. It is largely based on the Coleman *et al.* classification both in terms of nomenclature and approach and the most recent version published in the IUPHAR Receptor Compendium (Coleman *et al.*, 1994b) and summarised in Table 1.1.

1.2.3 Pharmacological characterization of receptors

Receptors can be classified on the basis of a variety of criteria; molecular information about the receptors themselves and their genes; second messenger/signal transduction pathways and by their pharmacological profiles. The most informative and accessible rationale in the field of prostaglandin research, as can be observed from the previously described characterization of prostanoid receptors, is the pharmacological classification of receptors. This method of receptor classification relies upon the interpretation of the effects of agonists and antagonists on the functional response of the tissue under investigation. A variety of methods can be adopted in the pharmacological classification of receptors but their usefulness is dependent on the availability of selective agonists and antagonists. The approaches undertaken in the present study are outlined below:

1.2.3.1 Receptor classification using relative agonist and antagonist potency ratios

When the rank order of the potency of a series of agonists in two different tissues is

found to be similar, the presence of the same receptor in both tissues is indicated, as is the case when an antagonist exhibits a similar potency. If two tissues express different receptors, a difference in the relative agonist potency ratio and/or antagonist potency would be expected. More quantitative and unequivocal evidence would be an estimation of the affinity constant from its pA_2 value (the negative logarithm to the base 10 of the concentration of the antagonist which causes a two-fold shift of the control agonist concentration-effect curve). If an antagonist is competitive (and reversible), this value is equal to the affinity constant of that antagonist at that receptor , that is, when the slope is unity in the corresponding Schild plot (Jenkinson *et al.*, 1995). However, it is possible for an antagonist to exhibit the same affinity at two different receptors, hence the ideal situation would be to have data with both agonists and antagonists.

1.2.3.2 Quantitative analyses of agonist concentration-effect data

The overall response induced by an agonist in a tissue is determined by two properties; its affinity for the receptor (how well it binds to the receptor) and its efficacy (the ability of the resultant agonist-receptor complex to elicit a response in that particular tissue). These parameters provide more quantitative information about the agonist and the complementary receptor and may provide more effective direction of chemical effort in the synthesis of novel agonist probes for a given receptor with the required properties, that is; affinity and efficacy which govern potency, and selectivity. Furthermore, estimates of agonist efficacy could increase our understanding of the transduction pathway linking activation of the receptor to the observed response, such as amplification of the initial signal through multiple biochemical coupling steps. In the case of the neutrophil, the range of functions which can be modulated by these pharmacological agents, the degrees of inhibition, and the quantitation of their effects on second messengers would provide knowledge of the 'cross-talk' between stimulatory and inhibitory signals within the neutrophil and how they influence cellular function.

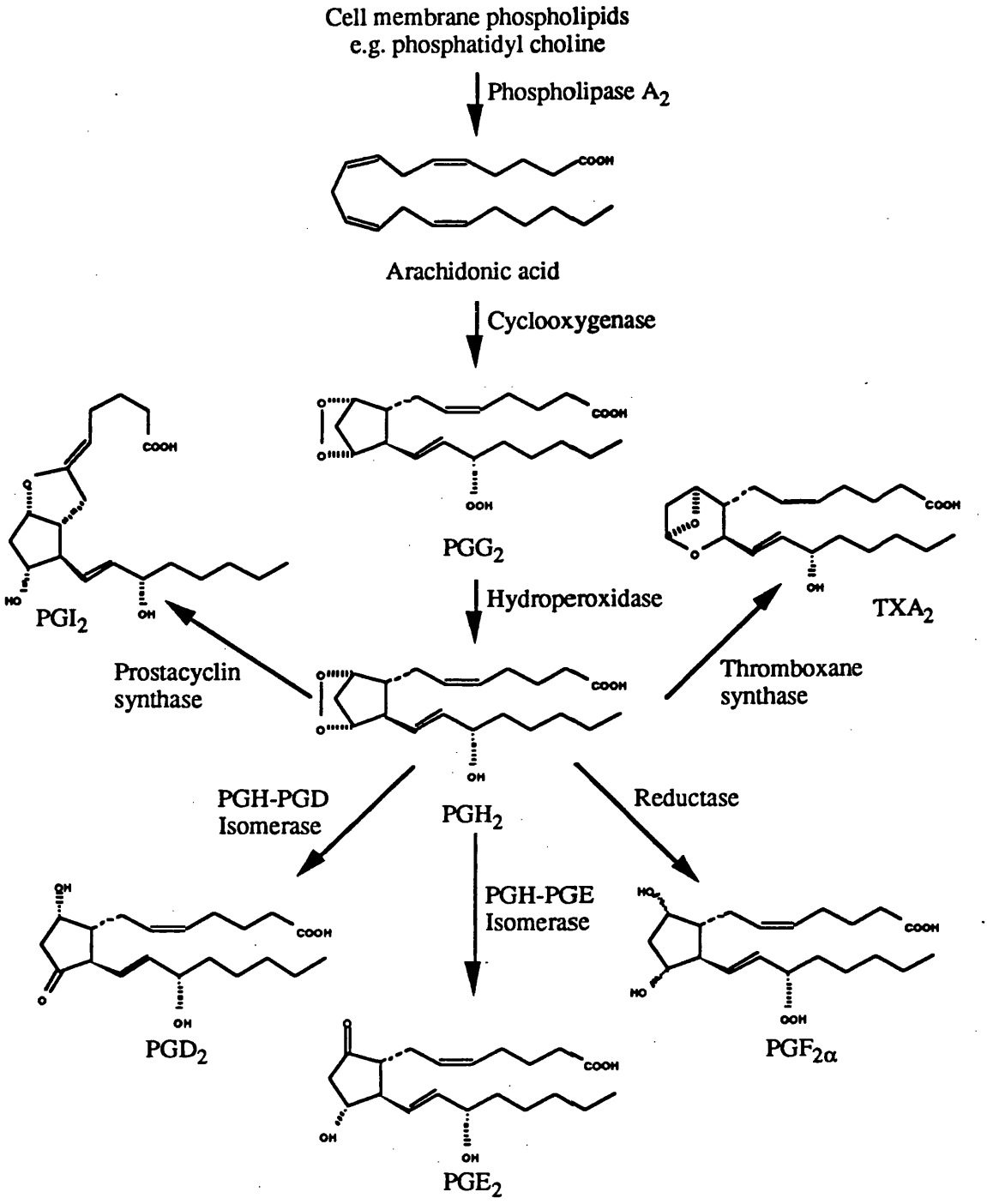


Fig 1.2 Synthetic pathway for PGE_2 and other prostaglandins from arachidonic acid.

Receptor Type	Endogenous Ligand	Selective Agonist	Selective Antagonist
DP	PGD_2	BW 245C ZK 110841	BW A868C AH 6809
EP (EP_1, EP_2, EP_3, EP_4)	PGE_2	See Table 3.1	See Table 3.1
FP	$PGF_{2\alpha}$	Fluprostenol Cloprostenol Prostalene	None
IP	PGI_2	Cicaprost Iloprost Octimibate	None
TP	TXA_2	U44609 U46619 SQ 26655	AH23848B GR32191B EP 092

Table 1.1 Classification of prostanoid receptors. Extracted from the IUPHAR Classification of Prostanoid Receptors (Coleman *et al.*, 1994). Prostanoid EP receptors discussed in further detail in Chapter 3.

1.3 Characterization of neutrophil inhibitory receptors using superoxide generation as a functional assay

1.3.1 Requirements of a functional assay for the characterization of neutrophil inhibitory receptors

In order to characterize receptors mediating inhibition of neutrophil activation, a suitable functional assay is required which fulfils certain criteria:-

1. The assay system must be sensitive to inhibition by the agents under investigation.

2. The functional assay should be simple, reproducible, of short duration and easily quantified to reduce the likelihood of degradation of the agents being studied.

3. It also desirable that the biological response used in the assay be physiological relevant, considered pro-inflammatory and destructive, and its suppression considered to be anti-inflammatory.

Most *in vitro* neutrophil activation assays occur rapidly and last a few minutes, such as superoxide generation, degranulation, polarisation and phagocytosis. Others, for instance, chemotaxis and IL-8 synthesis, have a considerably longer time course (hours). However the time course of neutrophil responses is also dependent on the type of stimulus used. In general, soluble stimuli, like C5a and fMLP, elicit more rapid neutrophil responses than particulate stimuli, including immune complexes and opsonised particles. Particulate stimuli require ingestion to activate neutrophils, whilst soluble stimuli activate neutrophils directly via plasma membrane receptors.

Superoxide generation in neutrophils is rapidly induced by fMLP and has been widely used for investigating the inhibitory effect of adenosine receptor (Cronstein *et al.*, 1983) , β-adrenergic receptor (Lad *et al.*, 1985) and histamine receptor (Burde *et al.*, 1990) agonists, in addition to prostaglandins (Gryglewski *et al.*, 1987). Superoxide

generation is easily and more accurately quantified spectrophotometrically by measuring the reduction of ferricytochrome C (Nauseef *et al.*, 1983). Other less direct protocols measure hydrogen peroxide, a distal product of the same pathway, e.g. with scopoletin (reviewed by Hayes & Fletcher, 1990). Thus, fMLP-stimulated superoxide generation using ferricytochrome C was chosen in the present study for the characterization of inhibitory prostaglandin receptors in human neutrophils.

1.3.2 Human neutrophil NADPH-oxidase - the superoxide anion generation complex

NADPH-oxidase catalyses the one electron reduction of oxygen to superoxide (O_2^-) using NADPH as the electron donor (reviewed by Babior, 1978 a,b). The formation of superoxide anions through this system is the initial step leading to the generation of all reactive oxygen metabolites by neutrophils, such as hydrogen peroxide (H_2O_2), the hypochlorite ion (ClO^-) and the hydroxyl radical ($OH\cdot$). In the resting neutrophil, the NADPH-oxidase complex is disassembled with components dispersed in the plasma membrane and in the cytosol. On stimulation, the cytosolic components translocate to the membrane to assemble the active oxidase. Electrons are donated by NADPH to a flavoprotein functioning as NADPH dehydrogenase and passed on to the terminal oxidase, cytochrome b_{558}, which reacts with O_2 to produce O_2^- (Fig 1.3).

The stoichiometry of the formation of superoxide anions is:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADPH^+ + H^+$$

The oxidase components are the best characterized of the NADPH-oxidase complex; cytochrome b_{558} is a heterodimer consisting of a small haeme carrying subunit (α chain) of 22 kDa ($p22^{phox}$) and a large highly glycosylated subunit (β chain) of 91kDa ($gp91^{phox}$). A 22kDa G protein, Rap1A, is closely associated with cytochrome b_{558} (Quinn *et al.*, 1989) and colocalises with cytochrome b_{558} in the plasma membrane and also within specific granules (Quinn *et al.*, 1992), which suggests that this complex

may be formed during activation. The two cytosolic components, p47^{phox} and p67^{phox}, with molecular weights of 47kDa and 67kDa respectively are essential for oxidase activity. Protein kinase C-dependent phosphorylation of p47^{phox} correlates with the association of p47^{phox} with the cytoskeleton and the translocation of p47^{phox} and p67^{phox} to the plasma membrane (Nauseef *et al.*, 1991). Protein kinase C may represent an activation signal used by receptor-mediated stimuli e.g. fMLP to induce NADPH-oxidase activation. In chronic granulomatous disease (CGD), the NADPH-oxidase is defective and the leukocytes are unable to generate superoxide anions resulting in abnormal responses. The cause of the defect are mutations of the α ($\leq 5\%$) or β ($\approx 55\%$) subunit of cytochrome b_{558} or the cytosolic factors p47^{phox} ($\approx 35\%$) and p67^{phox} ($\leq 5\%$) (reviewed by Morel *et al.*, 1991), thus demonstrating that all four of these components are required for the formation of the active oxidase. The NADPH dehydrogenase component(s) is less well characterized, and the nature and identity of this electron carrier linking NADPH to cytochrome b_{558} is more controversial. It has been identified as a FAD-binding flavoprotein, and some evidence suggests that the NADPH dehydrogenase is localised in the cytosol in resting cells and its translocation to the membrane during activation is dependent on the presence of p47^{phox} (Umei *et al.*, 1991).

1.3.3 Reactive oxygen metabolites formed from superoxide anions by neutrophils

Superoxide anions generated by NADPH-oxidase can be modified by neutrophils to form different and more microbicidal reactive oxygen metabolites e.g.hydrogen peroxide (H_2O_2), hypochlorite anions (ClO^-), the hydroxyl radical ($OH\cdot$) and chloramines.

Hydrogen peroxide is formed either by the spontaneous dismutation of superoxide anions or can be catalysed by superoxide dismutase:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Myeloperoxidase released from the neutrophil azurophil granules catalyses the oxidation of halide ions by hydrogen peroxide into hypohalite ions which are are also bactericidal. As the most abundant halide anion in neutrophils is the chloride ion Cl^-, the hypochlorite ion is formed:

$$Cl^- + H_2O_2 \rightarrow ClO^- + H_2O$$

These hypochlorite ions react with amines to form chloramines which are powerful oxidising agents and the most toxic ROM derived from the respiratory burst. In addition to this the hydroxyl radical, OH· may also be formed from O_2^- (Weiss *et al.*, 1977 and Tauber *et al.*, 1977) although the precise mechanism is unclear. Superoxide anions may also react with NO to form peroxynitrite anions ($ONOO^-$), which may generate reactive hydroxyl and nitrogen dioxide radicals (Evans, 1994).

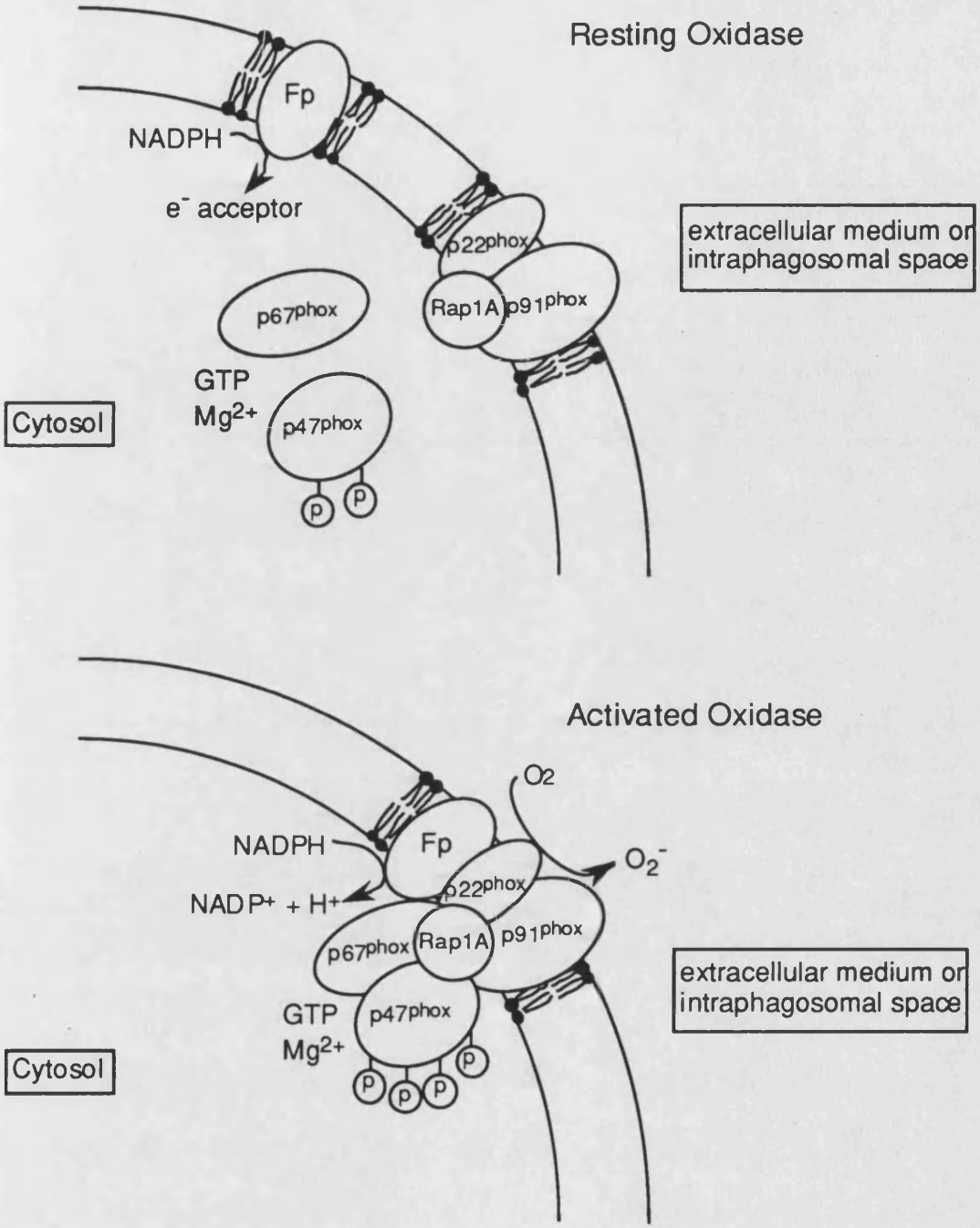


Fig 1.3 Organisation of the NADPH-oxidase system of the human neutrophil: transition from the resting state to the activated state. In unstimulated neutrophils (resting state) the oxidase is dormant and dissociated with components distributed in the cytosol and plasma membrane. Upon stimulation (activated state), the soluble components, p47^{phox} and p67^{phox}, translocate to the plasma membrane and associate with the NADPH flavodehydrogenase (Fp) and cytochrome b_{558} (p22^{phox} and p91^{phox}). Rap1A, a ras-related G protein, is closely associated with cytochrome b_{558}.

1.4 Aims of this thesis

The aims of this thesis are to pharmacologically characterize the prostanoid EP receptors mediating inhibition of leukocyte activation *in vitro* using the following approaches:

1. Characterize the inhibitory prostanoid EP receptors of human neutrophils using fMLP-stimulated superoxide generation as the functional assay with a range of selective agonists and antagonists.

2. Determine whether cAMP elevation is the second messenger pathway utilized by this inhibitory prostanoid EP receptor in the human neutrophil.

3. Further determine whether EP receptor agonist-mediated cAMP elevation modulation of $[Ca^{2+}]_i$ is involved in the regulation of human neutrophil activation by comparing the effects of EP receptor agonists and phosphodieserase inhibitors on fMLP-stimulated increases in $[Ca^{2+}]_i$.

4. Investigate the inhibitory profile of PGE_2 on human neutrophil activation by comparing its potency and efficacy as an inhibitor of superoxide generation, degranulation and shape change stimulated by a range of agonists.

5. Further determine the profile of the inhibitory effect of PGE_2 on other leukocytes by characterizing the prostanoid EP receptors present on human peripheral blood monocytes and undifferentiated HL-60 cells (a human promyelocytic cell line) and the neutrophils of another species (rabbit).

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Compounds

2.1.1.1 Prostanoids

Bayer plc supplied:-

Butaprost: methyl(1R,2R,3R)-3-hydroxy-2-[(1E,4R)-4-hydroxy-4-(1-propylcyclobutyl)-1-butenyl]-5-oxocyclopentaneheptenoate

Glaxo Wellcome Research generously supplied:-

AH 6809: 6-isopropoxy-9-oxoxanthene-2-carboxylic acid

AH13205: (+/-)-trans-2-4[4-(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoic acid

AH23848B: (1α[z],2β,5α)-(±)-7-(5-[{(1,1'-biphenyl)-4-yl}methoxy]-2-[4-morpholinyl]-3-oxocyclopentyl)-4-heptenoic acid

BW 245C: 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl)hydantoin

BW A868C: 3-benzyl-5-[6-carboxyhexyl]-1-[2-cyclohexyl-2-hydroxyethylamino]hydantoin

GR 63799X: (1R-[1α{Z},2β{R*},3α]-4-(benzoylamino)phenyl-7-(3-hydroxy-2-[2-hydroxy-3-phenoxypropoxy]-5-oxocyclopentyl)4-heptenoate

GR 32191B: (IR-[1α{Z},2β,5α])-(+)-7-(5-[{1,1'-biphenyl}-4-ylmethoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl)-4-heptanoic acid

Ono Pharmaceuticals supplied:-

Viprostol: (±)-methyl(Z)-7-[(1R,2R,3R)-2-[(E)-(4RS)-4-butyl-4-hydroxy-1,5-hexadienyl]-3-hydroxy-5-oxocyclopentyl]-5-heptenoate

Rhône-Poulenc Rorer supplied:-

M&B 28,767: (±)15α-hydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranorprost-13-trans-enoic acid

Schering AG supplied:-

Cicaprost: [2-[(2E,3aS,4S,5R,6aS)-hexahydro-5-hydroxy-4-[3S,4S)-3-hydroxy-4-methyl-1,6-nonadiynyl]-2-(1H)-pentalenylidene]ethoxy]acetic acid

Iloprost: (E)-(3aS,4R,5R,6aS)-hexahydro-5-hydroxy-4-[(E)-(3S,4RS)-3-hydroxy-4-methyl-1-octen-6-ynyl]-$\Delta^{2(1H),\delta}$-pentalenevaleric acid

Sulprostone: (Z)-7-[(1R,2R,3R)-3-hydroxy-2-[(E)-(3R)-(3-hydroxy-4-phenoxy-1-butenyl)]-5-oxo-cyclopentyl]-N-(methylsulfonyl)-5-heptenamide

Searle supplied:-

Misoprostol: (±)-methyl(1R,2R,3R)-3-hydroxy-2-[(E)-(4RS)-4-hydroxy-4-methyl-1-octenyl]-5-oxocyclopentaneheptanoate (purchased from Cascade 1994 onwards).

SC 19220: 1-acetyl-2-(8-chloro-10,11,dihydro-benz[*b*,*f*][1,4]oxazepine-10-carbonyl)hydrazine

All other prostaglandins were bought from Cascade Biochem Ltd, Reading. Structures of most widely used prostanoids shown in Figs 2.1, 2.2 and 2.3.

2.1.1.2 Adenosine receptor ligands

Sigma supplied:-

Adenosine

NECA: 5'-*N*-carboxamidoadenosine

2.1.1.3 Phophodiesterase Inhibitors (PDEIs) (Structures shown in Fig 2.4)

Sigma supplied:-

IBMX: 3-isobutylmethyl xanthine

Research Biochemicals International (RBI®) supplied:-

RO 20-1724: (4-(3-butoxy-4-methoxy-4-methoxybenzyl)-2-imidazolidinone)

Rolipram: 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone synthesised "in house" at Astra Charnwood.

Calbiochem supplied:-

Milrinone: 1,6-dihydro-2-methyl-6-oxo(3,4'-bipyridine)-5-carbonitrile

2.1.1.4 Other reagents

Cytochalasin B, ferri-cytochrome C (type IV), digitonin, dimethyl sulphoxide (DMSO), econazole, formyl-methionyl-leucyl-phenylalanine (fMLP), gluteraldehyde, MOPS (3-[N-morpholino]propanesulfonic acid), superoxide dismutase (SOD), human recombinant C5a, forskolin, p-dinitrophenyl-β-d-glucuronide, Percoll, Triton X-100 and zymosan A were obtained from Sigma (Poole, Dorset). H-89 {N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl}, and SQ 22,536 (9-(tetrahydro-2'-furyl)adenine) obtained from Calbiochem. Fura-2am was supplied by Molecular Probes Inc (Eugene, Oregan). Human recombinant IL-8 (72 amino acid form) was obtained from Advanced Protein Products, Brierley Hill, West Midlands, and C_{16} platelet activating factor (PAF) from Cascade Biochem Ltd, Reading. Sterile 0.9% saline and 3.8% sodium citrate solutions were from Phoenix Pharmaceuticals. Heparin (Monoparin) and EDTA tubes (Monovette) were bought from Evans. Dextran T500 was bought from Pharmacia. Polymorphprep™, Lymphoprep™ and Nycoprep™ 1.068g from Nycomed U.K., Birmingham and Monopoly Resolving Medium from Flow Laboratories. Hank's buffered salt solution (HBSS) and all culture products were from Imperial and Gibco. Phosphate buffered saline (PBS) tablets from Oxoid. Sagatal and Euthatal anaesthetics (both pentobarbitone sodium) were obtained from Rhône Mérieux and Saffan™ (an anaesthetic steroid) was supplied by Pitman-Moore. Sodium caseinate

was purchased from Difco. All other laboratory reagents were of analytical grade and obtained from Fisons Scientific Equipment.

2.1.2 Stimuli and priming agents

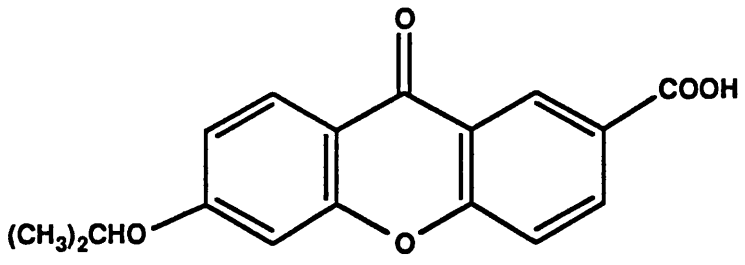
Human recombinant C5a stock solutions (10^{-4}M) and IL-8 stock solutions (10μg ml^{-1}) were prepared in 0.1% HSA in PBS, whilst C_{16} PAF was dissolved in distilled water at 10^{-2}M. All stocks were stored at -20°C and dilutions made in buffer containing 0.1% HSA. fMLP stock solutions (10^{-2}M in DMSO) were also stored at -20°C.

2.1.3 Opsonisation of zymosan

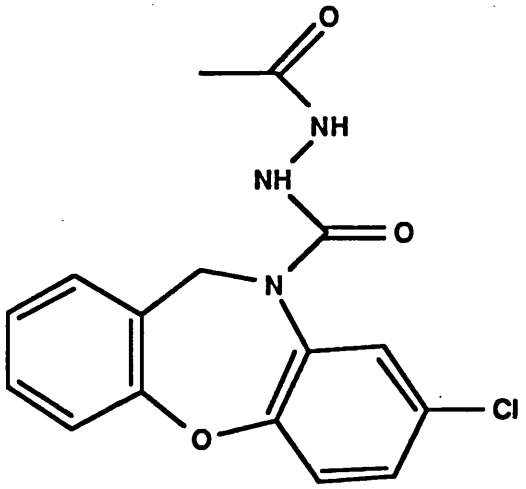
Fresh human serum was prepared by placing uncoagulated blood in a clean glass universal bottle (up to 20ml per bottle) which was allowed to stand for 2h at room temperature. The clot was freed ('rimming') from the walls of the vessel with an autoclaved glass Pasteur pipette at 1h and 2h to allow the clot to contract. The universal was centrifuged (1000g, 10 min, 20°C) and the serum placed in a sterile 50ml polypropylene centrifuge tube.

Zymosan A (yeast cell wall particles from Saccharomyces cerevisiae) was suspended in sterile PBS (5mg ml^{-1}), vortex-mixed and the suspension passed through a 23 gauge needle to disperse aggregates. After centrifugation (1000g, 5 min, 20°C) the zymosan was resuspended in freshly prepared human serum at 15mg ml^{-1} with rigorous pipetting in a sterile 13.5ml conical polystyrene centrifuge tube and incubated in a shaking water-bath at 37°C for 30 min. The suspension was washed twice by centrifuging (1000g, 5 min, 20°C) resuspending in fresh PBS and finally resuspended in PBS at 10mg ml^{-1} and the suspension once again passed through a 23 gauge needle. This suspension as stored at 4°C until required, and vortex-mixed before diluting. The opsonised zymosan was viable when stored up to 3 days at this temperature and merely passed through a 23 gauge needle before use.

AH 6809



SC 19220



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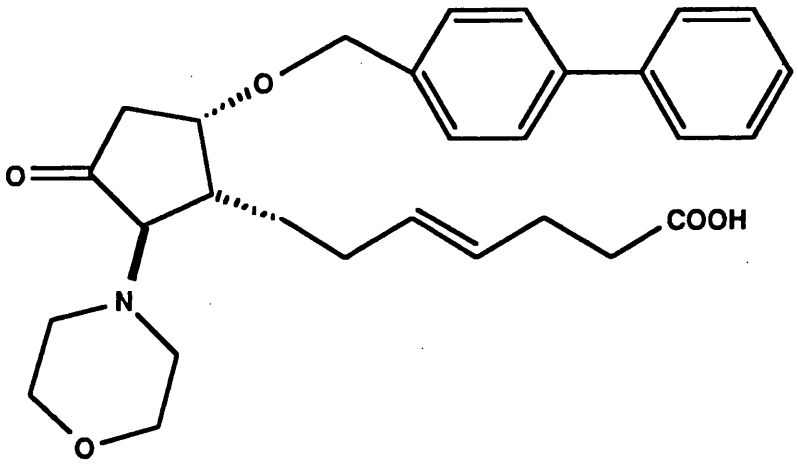
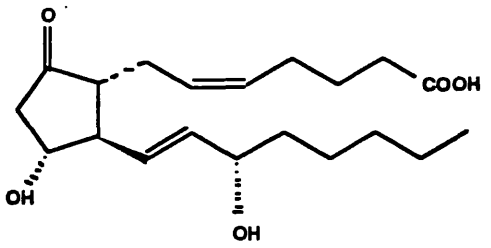


Fig 2.1 Structures of Prostanoid EP receptor antagonists

PGE$_2$



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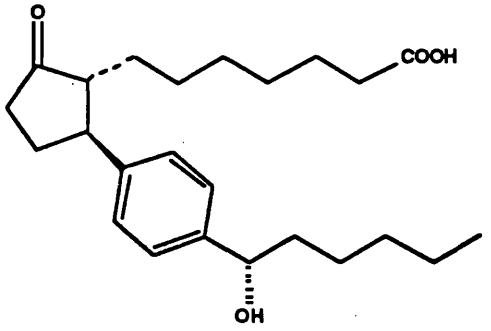
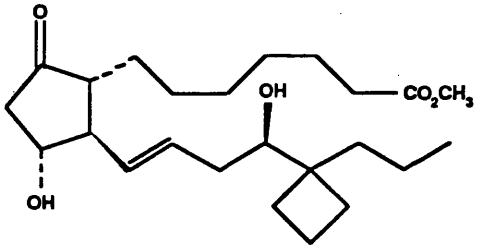
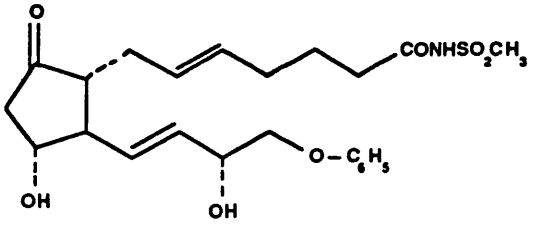


Butaprost



Sulprostone



GR63799X

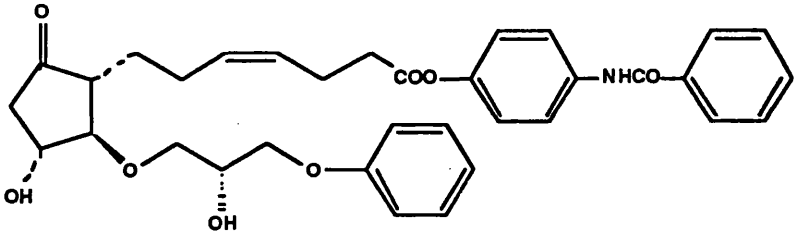
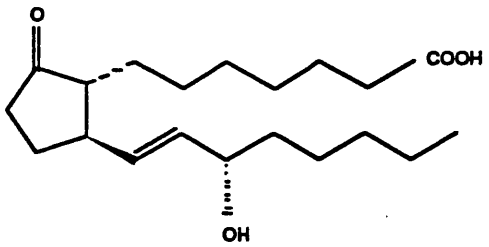
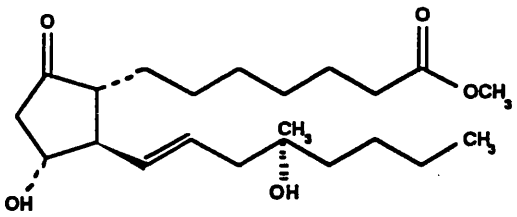


Fig 2.2 Structures of PGE_2 and selective prostanoid EP receptor agonists

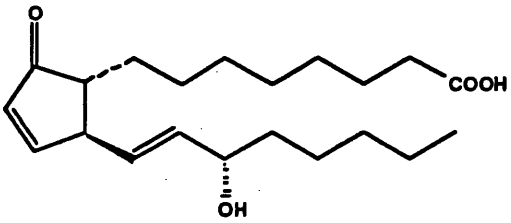
11-deoxy PGE_1



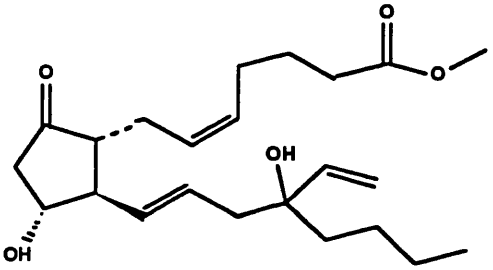
Misoprostol



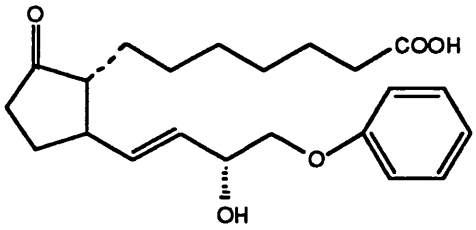
PGA_1



Viprostol



M&B 28,767



16,16-dimethyl PGE_2

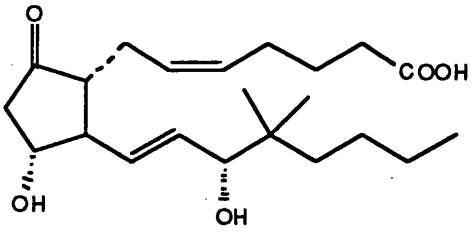
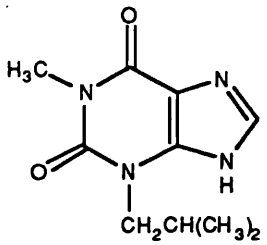
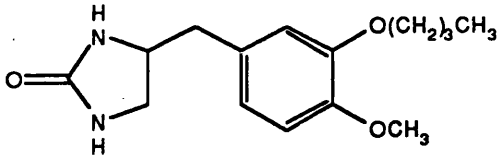


Fig 2.3 Structures of prostanoid EP receptor agonists

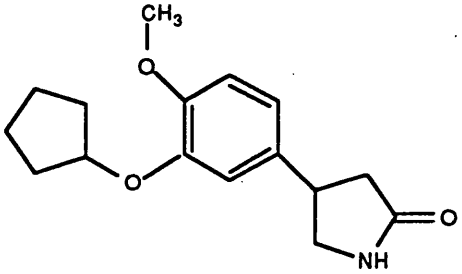
IBMX



RO 20-1724



Rolipram



Milrinone

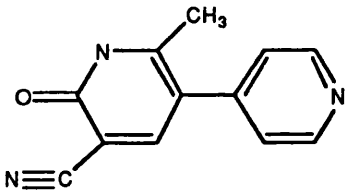


Fig 2.4 Structures of Phosphodiesterase Inhibitors

2.2 Preparation of human neutrophils

Neutrophils were isolated from blood donated by healthy male and female members of the human volunteer panel of Astra Charnwood or members of the Department of Pharmacology, School of Pharmacy and Pharmacology, University of Bath. Venous blood (100ml from each donor) was collected in 50ml syringes from the antecubital vein with 21 gauge venepuncture sets (Vygon) and anticoagulated with heparin (10U ml^{-1} blood, Monoparin) in the Department of Clinical Pharmacology, Astra Charnwood, Research and Development Laboratories, Loughborough or the Medical Centre, University of Bath.

The neutrophils were isolated using aseptic techniques in Class II cabinets. Initial experiments used Monopoly Resolving Medium as the separating medium; however due to its inconsistent supply its use was discontinued and Polymorphprep was used in all subsequent experiments.

2.2.1 Polymorphprep /Mono-Poly Resolving Medium

Heparinised blood was layered onto Monopoly Resolving Medium (MPM) in the ratio of 4ml blood : 3ml MPM in 13.5ml polystyrene conical centrifuge tubes (Sterilin) and centrifuged (300g, 20°C) for 45 min. With Polymorphprep as the separating medium, 5ml heparinised blood was layered onto 3.5ml Polymorphprep in 13.5ml polystyrene conical centrifuge tubes (Sterilin) and centrifuged (450-470g, 20°C) for 25 min. Both of these methods resulted in two bands of leukocytes; the upper band contained mononuclear cells and platelets whilst the lower band was composed of granulocytes, suspended over a pellet of erythrocytes. Thus, both allowed the one-step separation of polymorphonuclear leukocytes (PMN) from human blood.

The mononuclear leukocyte band was discarded with the plasma and then the lower granulocyte band from tubes from individual donors were collected and pooled in 4x 50ml conical polypropylene centrifuge tubes and diluted with an equal volume of RPMI

1640 (buffered with sodium bicarbonate at 2g l^{-1}) and supplemented with penicillin (100U ml^{-1}) and streptomycin (100µg ml^{-1}). These tubes were centrifuged (400g, 20°C) for 10 min, the supernatant discarded and contaminating erythrocytes removed by hypotonic lysis (20ml cold 0.2% NaCl, 30s followed by 20ml 1.6% NaCl). Following centrifugation (400g, 20°C) for 10 min, the cell pellet was gently resuspended in 5ml RPMI 1640. The granulocytes were counted and viability assessed by Trypan Blue exclusion (viability >99%). The purity of the cell preparation was examined microscopically on cytospin preparations with Wright's stain and was typically >95% neutrophils, the remainder consisted of eosinophils and monocytes. There was no apparent difference in the purity or function of the cells isolated using Monopoly Resolving Medium and Polymorphprep. The cells were maintained at room temperature rolling on a Coulter Roller in RPMI 1640 at $1x10^7$ ml^{-1} or less.

2.2.2 Lymphoprep Method

Venous blood was taken (100ml) and heparinised as described in 2.2 and then centrifuged (400g, 6 min) at room temperature. The platelet rich plasma was removed and the residual pellet was diluted to 200ml with RPMI 1640 (HEPES buffered, supplemented with penicillin 100U ml^{-1}, streptomycin 100µg ml^{-1} and fungizone 1µg ml^{-1}) in a 75cm^2 culture flask and mixed gently. The diluted blood was gently layered on to Lymphoprep in 50ml conical centrifuge tubes in the ratio of approximately 30ml diluted blood : 15ml Lymphoprep and centrifuged for 30 min (250g) at room temperature. The single band of mononuclear cells and all the supernatant above the erythrocyte pellet were removed with a plastic Pasteur pipette, and the pellets gently mixed and pooled (2 into 1). Warmed 6% dextran (500,000 MWt.) in saline (5ml) was added to each tube, the volume made up to 50ml with saline and the contents gently and thoroughly mixed and allowed to stand for 30 min at room temperature. The leukocyte rich supernatant above the sedimented erythrocytes was collected, centrifuged (400g, 10 min, 20°C) and the contaminating erythrocytes removed by hypotonic lysis (10ml ice-cold 0.2% NaCl for

30s followed by 10ml 1.6% NaCl). After centrifugation (400g, 10 min, 20°C), the cells were resuspended in 5ml HBSS (Ca^{2+}/Mg^{2+} free, pH 7.2-7.4, 0.1% BSA), counted and viability assessed microscopically by Trypan blue exclusion.

2.2.3 The Percoll Method

The method used in this study is based on that described by Haslett *et al.*, (1985), with modifications made by members of Division of Gastroenterology, University Hospital, Nottingham, who routinely use this method. Venous blood was taken as described in 2.2 and anti-coagulated with 3.8% sodium citrate (Phoenix Pharmaceuticals) in the proportion 1:10 v/v citrate to blood. A maximum of 40ml blood was collected in each 50ml polypropylene centrifuge tube, and the tubes were centrifuged (400g, 20 min, 20°C). The platelet rich plasma (PRP) was aspirated and centrifuged (1000g, 15 min, 20°C) to generate platelet poor plasma (PPP) which was put aside for making the Percoll gradients and resuspending the leukocytes. Meanwhile 6ml of warmed 6% dextran (MWt 500,000) in 0.9% saline was added to each cell pellet, the volume made up to 50ml with sterile saline and the contents of the tube was gently and thoroughly mixed by inversion and rocking. This was then allowed to stand vertically at room temperature for 30 min to sediment the erythrocytes. The leukocyte rich supernatant was removed and centrifuged (400g, 10 min, 20°C), and the cell pellet gently resuspended in 2-3ml autologous PPP in a 13.5ml polystyrene conical centrifuge tube. This was underlayered with 2ml freshly prepared 42% Percoll (previously diluted to 90% with sterile saline) in PPP which was in turn underlayered with 2ml freshly prepared 51% Percoll (previously diluted to 90% with sterile saline) in PPP using an autoclaved 223mm glass Pasteur pipette. The gradient was centrifuged (223g, 10 min, 20°C) and resulted in an upper band of mononuclear cells located at the plasma/42% Percoll interface and a lower band of PMN at the 42% Percoll/51% Percoll interface. The plasma and upper band was discarded and the PMN collected, diluted with PPP and centrifuged (400g, 10 min, 20°C). Any residual erythrocytes were removed by hypotonic lysis (10ml ice-cold 0.2% NaCl for 30s followed by 10ml 1.6% NaCl) and 2-3 drops of PPP. After centrifugation

(400g, 10 min, 20°C) the cells were resuspended in 5ml PPP and Trypan Blue exclusion used for microscopic determination of viability and counting.

2.3 Preparation of rabbit neutrophils

2.3.1 Isolation of rabbit peritoneal neutrophils

New Zealand White rabbits (male and female) weighing between 3.5-4.5kg were anaesthetised with 2ml Saffan™ (0.9% w/v alphaxolone and 0.3% w/v alphadolone acetate) administered in a bolus injection via an ear vein, and then given an intraperitoneal injection of 50ml 0.5% sodium caseinate in sterile PBS using a 19 gauge needle. The rabbits were allowed to regain consciousness and returned to their cages. The next day (approximately 18h later) the rabbits were killed with an intravenous bolus injection of Euthatal (pentobarbitone sodium, 400mg per rabbit) via an ear vein and the peritoneal cavity lavaged with 50ml PBS containing heparin (20U ml^{-1}). The cell suspension was centrifuged (400g, 10 min) and any contaminating erythrocytes were removed from the pellet by hypotonic lysis (10ml ice-cold 0.2% NaCl for 15s, followed by 10ml 1.6% NaCl). After centrifugation (400g, 10 min) the cells were resuspended in PBS containing 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$ at $2x10^7$ ml^{-1}. The cells were found to be >80% neutrophils in each case and viability as determined by Trypan Blue exclusion was >95%.

2.3.2 Isolation of rabbit peripheral blood neutrophils

New Zealand White rabbits (male, 3.5-4.5kg) were anaesthetised with Sagatal (pentobarbitone sodium, 150mg per rabbit) via an ear vein and anaesthesia maintained with supplements of Sagatal (pentobarbitone sodium, 15mg ml^{-1} in saline) via the same route. The throat was shaved and the carotid artery cannulated with a cannula (size 3Fg) flushed through with heparinised saline (10U ml^{-1}). Arterial blood was collected in 50ml polypropylene syringes containing 3.8% sodium citrate in the ratio 8ml citrate : 32ml blood and decanted into 50ml conical centrifuge tubes, after which the animal was

sacrificed with a bolus injection of Euthatal (400mg per rabbit, i.v.).

The neutrophils were isolated from the citrated arterial blood as described by Haslett *et al.* (1987). The tubes were centrifuged (300g, 20 min, 20°C) and the platelet rich plasma (PRP) generated was collected in 50ml centrifuge tubes and centrifuged (2000g, 15 min, 20°C) to generate platelet poor plasma (PPP). Meanwhile, 8ml of warmed 6% dextran (MWt 500,000) in sterile saline was added to the pellet of each 40ml citrated blood (in each tube) and the volume made up to 50ml with sterile saline. Each tube was then mixed gently and thoroughly by inverting and rolling and allowed to stand for 30 min (20°C) to sediment the erythrocytes. The leukocyte rich supernatant was collected in 50ml centrifuge tubes, centrifuged (400g, 20°C) and the pellet resuspended in 2-3ml autologous PPP and transferred to a 13.5ml polystyrene conical centrifuge tube. Each cell suspension was underlayered with 2ml 43% freshly prepared Percoll (diluted to 90% with saline) in PPP which was in turn underlayered by 53% Percoll (diluted to 90% with saline) in PPP. A gradient tube as just described, was prepared for every 40ml citrated blood collected and centrifuged (275g, 10 min, 20°C) and resulted in a band of mononuclear cells and platelets at the plasma/43% Percoll interface (top) and a wider band of PMN at the lower 43%/53% Percoll interface just above an erythrocyte pellet. The upper band of mononuclear cells and platelets was discarded and the lower band collected in a 50ml centrifuge tube and centrifuged (400g, 10 min, 20°C). Contaminating erythrocytes were removed by hypotonic lysis (10ml ice-cold 0.2% NaCl for 15s, followed by 10ml 1.6% NaCl) and 1-2 drops of autologous PPP. After centrifugation (400g, 10 min, 20°C) the cells were resuspended in 5ml autologous PPP, and a 20µl aliquot used for counting and assessment of viability by Trypan Blue exclusion. The cell preparations were examined microscopically and found to contain approximately 90% neutrophils.

2.4 Preparation of human monocytes

Venous blood was collected as described for the isolation of neutrophils except that the

blood was collected into 11 Monovette EDTA tubes (9ml per tube) and the isolation of monocytes performed aseptically in a Class II flow cabinet. 1 ml of 6% dextran (500,000 MWt.) in 0.9% saline was added to the contents of each tube and gently mixed. The tubes were allowed to stand vertically at room temperature for 30-40 min and the leukocyte rich plasma collected. 4.5-5.0ml of the leukocyte rich plasma was gently layered over 2.5ml Nycoprep 1.068 in 13.5ml polystyrene conical centrifuge tubes and the gradients centrifuged (600g, 15 min, 20°C).

The uppermost 1ml of plasma from each gradient was centrifuged in micro-centrifuge tubes (10,000rpm, 6 min, 20°C) to generate PPP. The broad band of monocytes at the plasma/Nycoprep interface was collected in a 50ml polypropylene centrifuge tube and centrifuged (400g, 10 min, 20°C). The cells were washed twice in 15ml 5% autologous PPP in 0.9% saline with centrifuging (50g, 10 min, 20°C) to reduce the number of contaminating platelets to final ratio of 1 monocyte:2-8 platelets. The purity of the leukocyte preparation was approximately 90% monocytes, the remainder of which were lymphocytes. The monocytes were maintained in 10ml 5% autologous PPP/saline rolling on a Coulter roller at room temperature until required.

2.5 Undifferentiated human promyelocytic leukaemia cells (HL-60)

HL-60 cells (ECACC, Porton Down) were maintained at a cell density of between $1x10^5$ and $1x10^6$ ml^{-1} in RPMI 1640 (buffered with sodium bicarbonate 2g l^{-1}, supplemented with penicillin 100U ml^{-1}, streptomycin 100µg ml^{-1} and 10% heat inactivated FCS) in 162cm^2 culture flasks (100ml) at 37°C and 5% CO_2 in an incubator.

2.6 Leukocyte functional assays

2.6.1 Superoxide assay

2.6.1.1 Human neutrophils

The neutrophils were removed from the Coulter Roller and centrifuged (400g, 20°C, 10

min) and resuspended at $1x10^7$ ml^{-1} in PBS containing 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$. The cells were preincubated with the antagonists at $5x10^6$ ml^{-1} on a coulter roller for 60 min at room temperature prior to addition of the agonists/inhibitors. The assays were performed in 75mm x 12mm polypropylene round bottomed centrifuge tubes with a final assay volume of 1ml ($1x10^6$ neutrophils ml^{-1}) in the presence of glucose (final assay concentration 11mM). The experiments were performed in the presence of cytochalasin B (fungal metabolite from Helminthosporium dematioideum), which disrupts the cytoskeleton of the cell and enables the release of superoxide anions into the supernatant. Cytochalasin B (final assay concentration 5µg ml^{-1}) was added simultaneously with the agonists (unless otherwise stated) at 37°C in a shaking water bath, followed by ferri-cytochrome C (horse heart, type VI, final assay concentration 0.085mM). 5 min after addition of the agonists, fMLP was added (final assay concentration 10^{-7}M) and incubated for a further 5 min. All treatments were performed in duplicate. The reaction was terminated by removing the tubes from the water bath and centrifuging the tubes (4°C, 10 min, 1000g).

An aliquot of each supernatant (300µl) was placed in 96 well cell culture plates and the absorbance read at 550nm on a Multiskan Mark II microplate reader (Flow Laboratories). The absorbances were zeroed on the SOD (90U ml^{-1}) samples which represented 100% inhibition. The effect of the inhibitors were determined as % inhibition of the fMLP response; and if antagonists were present, the antagonist/fMLP response was used as the reference.

2.6.1.2 Use of inhibitors in superoxide assay

The assay protocol used was as described in 2.6. Phosphodiesterase inhibitors and prostaglandin agonists or other inhibitors were simultaneously added (37°C), 5 min prior to fMLP stimulation.

For the experiments using divalent cation chelators, the cells were centrifuged (400g,

20°C, 10 min) and resuspended in 10ml PBS (Ca^{2+}/Mg^{2+} free) containing 1mM EGTA and left rolling on a Coulter Roller for 20 min at room temperature prior to centrifuging (400g, 20°C, 10 min) and resuspension in PBS (Ca^{2+}/Mg^{2+} free) for the assay. The cations where added to the appropriate tubes just prior to the addition of the neutrophils ($1x10^6$ ml^{-1} per sample) as were the chelators, and the assay performed as described in 2.6.1.1.

2.6.1.3 Superoxide generation stimulated by other agonists and priming agents

The stimulation period chosen for opsonised zymosan was 15 min at 37°C in the absence of cytochalasin B (5µg ml^{-1}), and 5 min for C5a in the presence of cytochalasin B (5µg ml^{-1}).

Priming of superoxide generation with PAF ($2x10^{-7}$M) used a 5 min preincubation period (37°C) prior to addition of the stimulus, with cytochrome C already present in the tubes but cytochalasin B was omitted as the priming effect was more pronounced in its absence.

2.6.1.4 fMLP-stimulated superoxide generation by rabbit neutrophils elicited from the peritoneal cavity and from peripheral blood

The superoxide assay was performed in the presence of cytochalasin B (5µg ml^{-1}) using 10^{-7}M fMLP as the stimulus with $2x10^6$ (elicited) or $1x10^6$ (peripheral blood) neutrophils ml^{-1} in each tube.

2.6.2 Measurement of cAMP generation

2.6.2.1 Human neutrophils

Human neutrophils were isolated as described in 2.2.1, and the cells from 2-3 donors were pooled just prior to the experiment. The neutrophils ($2x10^7$ ml^{-1}) were preincubated with the non-selective phosphodiesterase inhibitor IBMX ($5x10^{-4}$M) in

PBS/Ca/Mg and 11mM glucose for 5 min at 37°C prior to aliquoting (100µl) into pre-warmed 1.5ml microcentrifuge tubes containing 50µl stimulus in a shaking water-bath set at 37°C. The tubes were gently shaken for 5 min and then the reaction stopped by the addition of 50µl 20% perchloric acid. The tubes were removed from the water-bath, capped, vortex-mixed and then placed on ice for at least 15 min. After which the tubes were centrifuged for 30s (10,000g) and the supernatants (~200µl) removed into fresh 1.5ml microcentrifuge tubes which were capped and stored at -70°C until required for extraction of the cAMP. All treatments were performed in duplicate unless otherwise stated.

2.6.2.2 Undifferentiated HL-60 cells

For the generation of cAMP, cells were removed from the incubator and placed in 50ml polypropylene conical centrifuge tubes and centrifuged (200g, 5 min, 20°C) and washed once in RPMI 1640 (buffered with sodium bicarbonate 2g l^{-1}, supplemented with penicillin 100U ml^{-1}, streptomycin 100µg ml^{-1}) before resuspending in 5ml PBS/Ca/Mg. The cells were counted on a Technicon Junior cell counter (Bayer Diagnostics, Basingstoke) and viability determined by Trypan Blue exclusion (>90%). The cells were diluted to $2x10^7$ ml^{-1} and in some experiments incubated with antagonists at room temperature rolling in polystyrene 7ml bijoux for 25 min. Thereafter, IBMX was added and incubated with the cells for 5 min at 37°C. Aliquots of the IBMX/HL-60 suspension (100µl) were then added to pre-warmed 1.5ml micro-centrifuge tubes containing 50µl of vehicle of stimulus and incubated for a further 10 min. The final assay concentration of HL-60 cells was $1x10^6$ per determination and IBMX was $5x10^{-4}$M. The reaction was stopped by the addition of 50µl 20% perchloric acid (ice-cold) and the samples treated, stored and extracted as described for human neutrophils (2.6.2.1).

2.6.2.3 Human peripheral blood monocytes

The cells (in 5% autologous PPP/saline) were centrifuged (50g, 10 min, 20°C) and resuspended in PBS/Ca/Mg at $4x10^6$ ml^{-1} and incubated for 25 min with the antagonists

rolling on a Coulter roller at room temperature. IBMX (final assay concentration $5x10^{-4}M$) and indomethacin (final assay concentration $3x10^{-6}M$) were added and incubated with the cells for 5 min at 37°C. Aliquots of the cell suspension (100µl, $1x10^5$ monocytes) were added to pre-warmed 1.5ml microcentrifuge tubes containing 50µl prostaglandin or vehicle and gently shaken on a water-bath (37°C) for 5 min. The reaction was stopped by the addition of 50µl 20% perchloric acid (ice-cold) and the samples treated, stored and extracted as described for human neutrophils (2.6.2.1).

2.6.2.4 Rabbit peripheral blood neutrophils

The assay was performed as described for human neutrophils i.e. 45 min preincubation with antagonists at room temperature, 5 min preincubation with IBMX (37°C) followed by stimulation with prostaglandins for 5 min (37°C) in a final volume of 150µl. The reaction was stopped by the addition of 50µl 20% ice-cold perchloric acid. However a lower concentration of cells ($0.5\text{-}1x10^6$ per determination was used as the yield from each rabbit was low, but the cells from individual rabbits were used and not mixed. The reaction was stopped by the addition of 50µl 20% perchloric acid (ice-cold) and the samples treated, stored and extracted as described for human neutrophils (2.6.2.1).

2.6.3 Extraction of cAMP

The cAMP samples were defrosted and allowed to come up to room temperature prior to extraction. An equal volume (200µl) of a 1:1 freshly prepared mixture of tri-n-octylamine and 1,1,2-trichloro-1,2,2-trifluoro-ethane (Freon) was added to each tube, capped and thoroughly vortex-mixed (at least 10s) and centrifuged for 1.5 min at 10,000g. This resulted in a trilayer of liquids in each tube, only the uppermost layer (extracted cAMP) was collected and placed in a fresh 1.5ml microcentrifuge tube. An aliquot of this extract was diluted in cAMP assay buffer and assayed for cAMP content.

2.6.4 Assay of cAMP

a) Initial experimental samples were assayed by ^3H-RIA (Amersham TRK.432) in

1.5ml micro-centrifuge tubes using 25µl volumes of samples, standards and reagents. The unbound ^{3}H-cAMP in 50µl of supernatant was measured after addition of 4ml Optiphase scintillant in a Packard Tricarb Scintillation Counter using a count time of 4 min.

b) Most of the experimental samples were assayed using ^{125}I-SPA RIA (Amersham RPA.538) a dual range scintillation proximity assay (SPA) system. All samples unless otherwise stated were assayed unacetylated. The RIA was performed in Packard 96 well plates using 50µl volumes of samples, standards and all reagents. The plates were heat-sealed, shaken and stood for 10-30h before counting on a Topcount counter with a count time of 5 min per well.

2.6.5 Fura-2 loading cells for spectrophotofluorimetry

These experiments were performed at the School of Pharmacy and Pharmacology, University of Bath, where the donors were volunteers from the members of the department and the neutrophils isolated using Lymphoprep as described in 2.2.2. Neutrophils were resuspended in HBSS (without Ca^{2+} or Mg^{2+} ions) containing 0.1% BSA (~$5x10^{7}$ ml^{-1}) and loaded with Fura-2am (2µM, 2.5µl of 5mM stock in DMSO per 5ml cell suspension) at 37°C for 30 min in the dark with intermittent shaking, essentially a modification of the method of Murphy *et al.* (1991). The cells were washed once in HBSS (pH 7.2-7.4, without Ca^{2+} or Mg^{2+} ions), viability assessed by Trypan Blue exclusion and resuspended in HBSS (pH 7.2-7.4, without Ca^{2+} or Mg^{2+} ions) containing 0.1% BSA supplemented with 100µM $CaCl_2$ at $5x10^{6}$ ml^{-1}. The cell suspensions were maintained on ice and aliquots taken for fluorimetry measurements after gentle mixing by inversion when required.

All the measurements of changes in intracellular calcium were performed on a PTI dual excitation dual emission fluorimeter (Photon Technology Instruments). A cuvette stirrer and a circulator were fitted, enabling the contents of the cuvettes (quartz) to be maintained

at 37°C and constantly stirred.

The experiments were performed at 37°C in a quartz cuvette using a constantly stirred 2ml aliquot of the neutrophil suspension ($5x10^6$ ml^{-1}) in the presence of 1mM $MgCl_2$ and 1mM $CaCl_2$ unless otherwise stated. The responses of the cells used at the beginning and the end of each experimental day were identical.

2.6.6 Determination of intracellular calcium levels using spectrophotofluorimetry

2.6.6.1 Measurement of neutrophil $[Ca^{2+}]_i$

The excitation wavelengths were set at 340nm (1A) and 380nm (2A), and emission was measured at 510nm. The fluorescence output was converted to $[Ca^{2+}]_i$ after calibration by using the equation described by Grynkiewicz *et al.* (1985) as shown below:-

$$[Ca^{2+}]_i = \frac{F - F_{min}}{F_{max} - F} \; x \; K_{Ca} \; x \frac{S_f 2}{S_b 2}$$

where F represents the fluorescence measurement (1A/2A ratio), F_{max} and F_{min} fluorescence in the obtained by the addition of 80µl of digitonin (4mg ml^{-1}, 70% ethanol/H_2O) in the presence of 1mM extracellular Ca^{2+} ions and the further addition of 80µl EGTA (100mM, preceded by 40µl 2M NaOH) respectively, K_{Ca} is the apparent dissociation constant for Ca^{2+} binding with Fura-2 and S_f2/S_b2 is the dilution correction factor determined from the ratio of the fluorescence of free Fura-2 and Ca^{2+}-bound Fura-2 measured using the 2A signal.

All of the experiments were performed in the presence of 1mM $MgCl_2$ and 1mM $CaCl_2$ unless otherwise stated. The cells were incubated with vehicle, PGE_2 or rolipram for 5 min at 37°C prior to stimulation with fMLP. Monitoring of $[Ca^{2+}]_i$ commenced 40s prior to the addition of fMLP to obtain a baseline measurement, and monitored for up to 5 min after the addition of fMLP.

2.6.7 Readdition of extracellular calcium ions

Experiments involving the re-addition of Ca^{2+} ions were based on the method described by Clementi *et al.* (1992). The cells were incubated with vehicle, PGE_2 or rolipram, 1mM $MgCl_2$, 0.1mM $CaCl_2$ and 1mM EGTA for 5 min at 37°C prior to the addition of buffer or fMLP (0.1μM). After which, 2mM $CaCl_2$ (in 20μl, 70s after buffer/fMLP) was added using a Hamilton syringe via the injection port to allow continuous recording.

2.6.8 Measurement of Mn^{2+} ion influx

Mn^{2+} influx was measured as the quenching of intracellular fura-2 excited at 360nm, the isobestic point for Ca^{2+} ions, and emission measured at 510nm (Murphy & Westwick, 1992). At this excitation wavelength, the presence of Ca^{2+} ions does not interfere with Mn^{2+}-induced quench of fura-2. Calibration was performed by expressing the agonist-induced response as percentage quench (AUC) compared to that achieved by ionomycin ($2x10^{-6}M$). $[Ca^{2+}]_i$ was measured simultaneously using the 340nm excitation and 510nm emission wavelengths. Vehicle, PGE_2 or rolipram were added and incubated at 37°C for 5 min prior to the addition of buffer, fMLP or ionomycin. $MnCl_2$ (0.1-1mM) was added 40s after the addition of the stimulus. The baseline was monitored for 40s prior to stimulation, and the signal monitored for up to 5 min after stimulation.

2.6.9 Degranulation as measured by β-glucuronidase release

Essentially the conditions used for the β-glucuronidase release assay from human neutrophils were identical to those used for superoxide generation except for the omission of ferri-cytochrome C from the assay system. After the samples were centrifuged (1000g, 10 min, 4°C), 100μl of the supernatants were removed for assay of β-glucuronidase content and the remainder was discarded. The cell pellets were lysed by the addition of 1ml 0.1% Triton X-100 (Sigma) in saline (0.1% BSA), vortex-mixed and

incubated at 37°C for 20 min. Cell debris was removed by centrifugation (1000g, 10 min, 4°C) and 100µl of the cell lysates were assayed for β-glucuronidase content.

Assay of β-glucuronidase enzyme activity in 100µl of supernatant or cell lysate was measured as the consumption of the substrate p-dinitrophenyl-β-d-glucuronide (600µl of 5mM stock) in 0.1M acetate buffer (pH 4.5) at 37°C overnight (at least 16h). The reaction was stopped by the addition of 300µl of 0.8M glycine buffer (pH 10.4). The samples were mixed and 300µl aliquots were placed in 96 well plates and the absorbance of the resultant colour (yellow dinitrophenol) was determined at 405nm using a Multiskan Mark II plate reader.

Results were expressed initially as % of total cellular β-glucuronidase content (A405nm in supernatant added to A405nm in pellet) released, effects of inhibitors expressed as % inhibition of fMLP response.

2.6.10 Neutrophil shape change (Polarisation)

2.6.10.1 Shape change assay

Neutrophils are exquisitely sensitive to becoming polarised and is the first visible sign of activation. This can occur as a result of many stimuli; contact with extremely low concentrations of priming agents, e.g. endotoxin (Haslett *et al.*, 1985). Rough handling, repeated temperature changes and prolonged contact with large numbers of other neutrophils on sedimentation can also induce a shape change in neutrophils. The preparation of neutrophils for shape change assays therefore requires extreme care and special procedures as described in 2.2.3.

The neutrophils were finally resuspended in HBSS (Ca^{2+}/Mg^{2+} free, 0.35% sodium bicarbonate, 10mM MOPS) at $1x10^7$ ml^{-1} for the assay.

The assay was based on the protocol described by Haston & Shields (1985) and used by

Cole and co-workers, Department of Gastroenterology, University of Nottingham. In this study, it was performed in triplicate in 12mm x 75mm polypropylene tubes with a final assay volume of 1ml HBSS containing 10mM MOPS, 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$, inhibitor, $1x10^6$ neutrophils and the stimulus. The neutrophils were added penultimately, the stimulus being the last addition. The tubes were capped and placed in an incubator (37°C) for 30 min. The reaction was stopped by the addition of 1ml 2.5% glutaraldehyde in PBS and the tubes left to stand at room temperature for at least 30 min. The tubes were vortex-mixed and then centrifuged (1000g, 10 min, 20°C) and the cells washed twice by resuspending the pellets in PBS, vortex-mixing and centrifugation (1000g, 10 min, 20°C). The cells were finally resuspended in 1ml PBS and stored at 4°C for up to 4 weeks, but vortex-mixed and allowed to come up to room temperature prior to analysis.

2.6.10.2 Microscopic determination of neutrophil shape change

Microscopic examination of the fixed neutrophils was determined essentially as described by Haston & Shields (1985). The cell suspensions were mounted in a double chamber Neubaur haemocytometer and 200 neutrophils from each sample were counted as either unpolarised (perfectly spherical) or polarised (exhibiting pseudopodia and or not spherical in shape), and % polarisation determined for each sample.

2.6.10.3 Flow cytometric determination of neutrophil polarisation

The samples were transferred to Becton Dickenson 12mm x 75mm polystyrene centrifuge tubes as recommended for use with the Coulter Elite flow cytometer. The flow cytometer settings used were as follows:-

The samples were vortex-mixed and when the flow rate of the cytometer was stabilised at between 60-120 events/second, forward light scatter and right angle light scatter data capture was initialised. Forward light scatter is a measurement of cell size which would increase when a neutrophil becomes polarised and exhibits pseudopodia, whilst right

angle (side) scatter indicates the degree of granularity of the cell which would not change. The data capture settings were gated on a negative control containing unpolarised cells (basal, approximately 4% polarised) and a positive treatment (maximally stimulated, fMLP $1x10^{-7}M$) which contained >95% polarised neutrophils and exclude erythrocytes and leukocytes other than neutrophils. This was especially important as fixing cells with gluteraldehyde can change the size of cells compared with fresh cells. The flow cytometer was programmed to analyse 6000 events within the whole (unpolarised and polarised) neutrophil region in terms of forward light scatter and the results for each sample were expressed as % polarised within the gated region.

There was a close degree of correlation between the 2 methods of counting (Fig 2.5, $r=0.994$, $p<0.05$, $n=33$ determinations (Cole and co-workers reported $r=0.994$, $p<0.001$). All samples were counted on the Elite Flow Cytometer which was more accurate; 6000 events counted instead of 200 per sample, it was also objective and was considerably less labour intensive and time consuming (1-2 min per sample compared to 10 min per sample if determined microscopically).

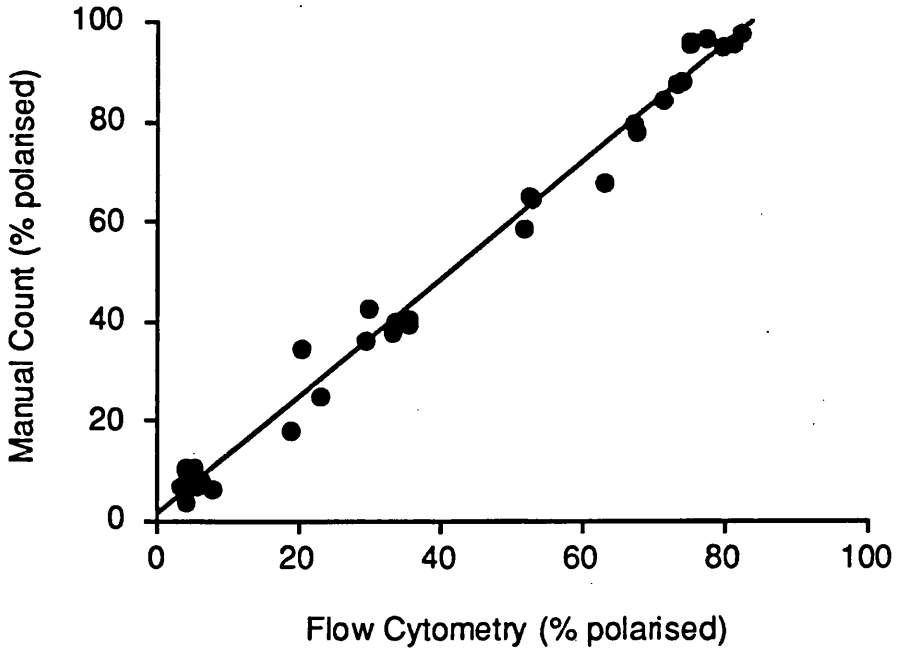


Fig 2.5 Correlation between manual and flow cytometric analysis of human neutrophil shape change. Comparison of the quantitation of human neutrophil shape change, as determined by manual counting (200 cells per sample counted) flow cytometric determination (6000 cells per sample) using the "Elite" flow cytometer (Coulter). Correlation determined using 33 samples, with correlation co-efficient of R=0.994.

2.7 Analysis of data

2.7.1 Analysis of agonist activity

Activity of agents were expressed as negative logarithm base 10 of the molar concentration of the agent producing 50% of own maximum response, i.e. a $p[A_{50}]$ value as described by Jenkinson *et al.* (1995). For agents which do not have a clear maximum effect, negative logarithm base 10 of the molar concentration of the agent giving 50% maximum response obtainable in the assay, i.e. $p[IC_{50}]$ or $p[EC_{50}]$ values were calculated (Jenkinson *et al.*, 1995).

2.7.2 The Operational Model of Agonism

The potency orders of the EP agonists in the superoxide and cAMP assays could not be compared directly as some of the agonists were partial in the cAMP assay. The effects of mathematically increasing receptor reserve were simulated using the Operational Model (Leff *et al.*, 1990) in both assays in order to compare agonist potency orders.

The mathematical form of the Operational Model

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n}$$

The Operational Model is described by the above equation, with E representing the tissue response to a given agonist concentration [A]), K_A as the agonist equilibrium dissociation constant and τ defining the model definition of the efficacy of an agonist in a tissue for the agonist under study. E_m (the maximum pharmacological effect that can be generated in a particular system, i.e. by a "full agonist") and n (the slope of the transducer relation, a measure of the sensitivity with which a particular system transduces AR the agonist-receptor complex into E). Fig 2.6 (upper panel) shows the concentration-effect (E/[A]) curves for full and partial agonists in a low receptor reserve

system. If the receptor reserve is increased as shown in Fig 2.6 (lower panel), the maximum effect of the full agonist is unchanged as it is already maximal and the curve simply left-shifts. With the same increase in receptor reserve, the partial agonists are now able to achieve the same maximum effect of the full agonist and also shift to the left.

The affinity (pK_A) and efficacy (τ) of the partial agonists were estimated by applying the Operational Model (Leff *et al.*, 1990) by comparison of a partial agonist with a full agonist (i.e. PGE_2). An increase in receptor reserve was simulated by increasing (τ) by a factor of 100 for the partial agonists to render them full agonists and allowing the corresponding changes in $p[A_{50}]$ for the full agonists in both the superoxide and cAMP assays. The potency orders of the agonists in both assays were then compared.

2.7.3 Analysis of antagonist activity

E/[A] curve data from 5 experiments for PGE_2 or BW 245C inhibition of fMLP superoxide generation in the absence and presence of AH 6809 were fitted to the Hill equation (i) of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \qquad (i)$$

in which α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively. $[A_{50}]$ values were assumed to be $\log_{10}$-normally distributed and they were estimated as such and quoted as $p[A_{50}]$ ($-\log_{10}[A_{50}]$) values.

The E/[A] data describing the 5 curves were fitted to a modified form of the Schild equation (ii) (Black *et al.*, 1985), where $[A_{50}^c]$ is a control $[A_{50}]$, [B] is the concentration of AH 6809, K_B is its equilibrium dissociation constant and n is the Schild plot slope.

$$\log_{10}[A_{50}] = \log_{10}[A_{50}^c] + \log_{10}(1 + [B]^n / K_B) \qquad (ii)$$

Additional goodness-of-fit analysis was performed on the pK_B estimate for AH 6809 from the Schild analysis with the slope unconstrained and constrained to unity, using PGE_2 as the agonist as described by Leff & Giles (1992).

2.7.4 Statistical Analyses of Data

The data was analysed for statistical significance using paired Student's t-test unless otherwise stated. Significance was determined by 95% confidence limits i.e. $P<0.05$, and indicated in figures and tables as * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$. Probabilities are reported for each comparison with the control group, so type I error rates may exceed those reported in the text.

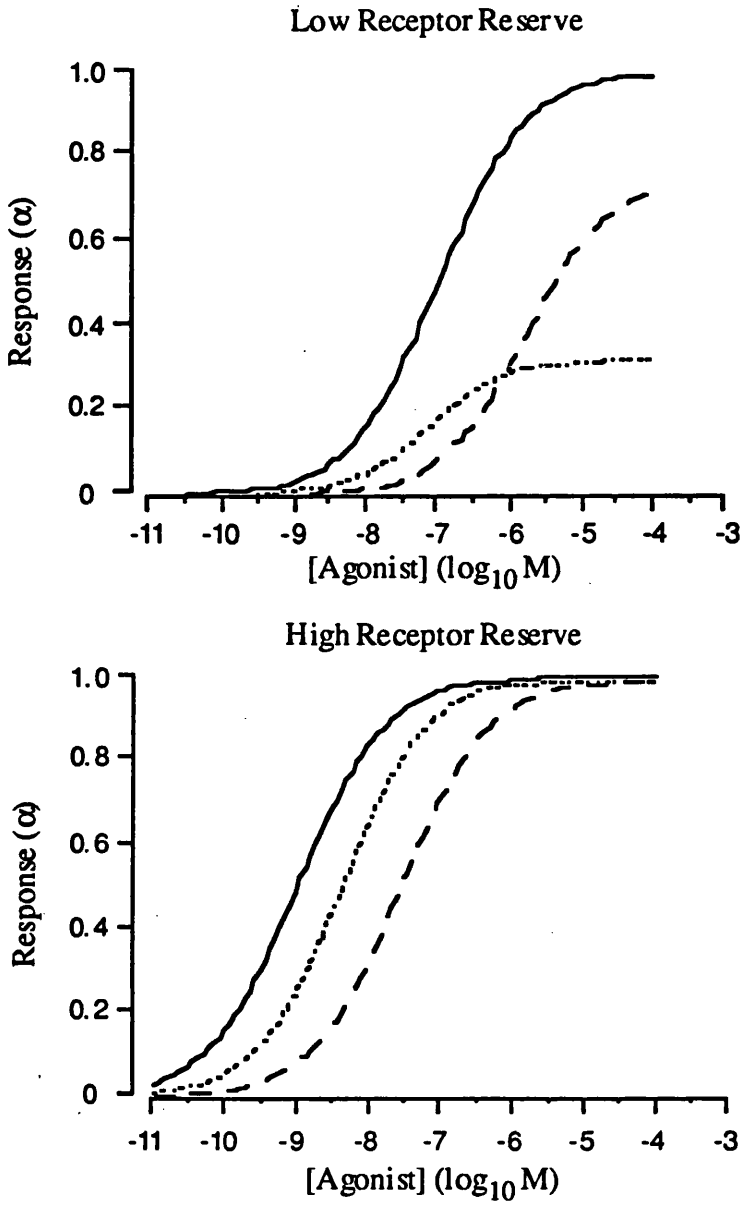


Fig 2.6 Illustration of the effect of receptor reserve on the responses of full and partial agonists according to the Operational Model. The upper panel shows the responses of a full agonist (continuous line) and two partial agonists with different efficacies (dashed and dotted lines) in a low receptor reserve system. When the receptor reserve is increased to a sufficient level as to allow the partial agonists to achieve the same maximum response as the full agonist as shown in the lower panel, the maximum response of the full agonist is unchanged as it is already maximal, and the E/[A] curve is simply left-shifted. Correspondingly, the E/[A] curves of partial agonists will also leftward shift.

CHAPTER 3

PHARMACOLOGICAL CHARACTERIZATION OF PROSTANOID EP RECEPTORS MEDIATING INHIBITION OF HUMAN NEUTROPHIL ACTIVATION

3.1 INTRODUCTION

3.1.1 Classification of E-Type prostaglandin (EP) receptors

Amongst the prostanoids, PGE_2 is arguably the prostanoid with the widest spectrum of biological activities; affecting the reproductive, cardiovascular, nervous and respiratory systems and the gastro-intestinal tract. Some of the most profound effects of PGE_2 are on inflammatory and immune responses. There has been much speculation as to the pathophysiological role of PGE_2 as both pro-inflammatory and anti-inflammatory effects have been described (Kunkel & Chensue, 1984). Despite the degree of interest in PGE_2, the EP receptors on the cells involved in inflammation and immunological responses are perhaps some of the least well characterized.

The classification of prostanoid EP receptors is based primarily on the effects of agonists and antagonists on smooth muscle preparations, as are most pharmacological receptor classifications. At present, the EP receptor is the only prostanoid receptor for which there is unequivocal evidence for subdivision and is outlined in Table 3.1.

The naturally occurring agonists, PGE_2 and PGE_1, are equiactive and neither shows selectivity between the EP receptor subtypes (Coleman *et al.*, 1987). One of the most definitive agonists is sulprostone (EP_1/EP_3 selective) (Bunce *et al.*, 1990), which is active at EP_1 and EP_3 receptors in the nanomolar concentration range. In contrast, sulprostone is greater than 1000 times less potent than PGE_2 at EP_2 receptors (Coleman *et al.*, 1987). Butaprost is also widely used, although it is less potent as an agonist (active in the micromolar concentration range), it is more selective, showing agonism only at the EP_2 subtype (Gardiner, 1986).

There is a lack of selective antagonists for the EP receptor subtypes, hence the characterization of prostanoid EP receptors relies heavily upon the use of agonist potency

orders. There are only antagonists described for the EP_1 receptor subtype, i.e. SC 19220 and AH 6809 show a similar activity to each other at EP_1, EP_2 and EP_3 receptors (Coleman *et al.*, 1987), antagonising EP_1 receptors with pA_2 values of 5.4 and 6.8 respectively. The pA_2 values for SC 19220 at both EP_2 and EP_3 receptors were <4.0, similarly the pA_2 values in the same tissues were <5.0 for AH 6809.

Receptor Subtype	Selective Agonist	Selective Antagonist	pA_2
EP_1	[1]Sulprostone	[6]SC 19220	5.2-5.6
		[6¶]AH 6809	6.4-7.0
EP_2	[2]AH13205/[3]Butaprost	None	
EP_3	[4]Sulprostone/[5]GR63799X	None	
EP_4	None	[7]AH23848B	5.4

Table 3.1 Classification of prostanoid EP receptor subtypes, selective agonists and antagonists. The data was obtained from the following publications; [1]Dong *et al.*, 1986, [2]Nials *et al.*, 1993, [3]Gardiner, 1986, [4]Coleman *et al.*, 1987, [5]Bunce *et al.*, 1990, [6]Coleman *et al.*, 1985, [7]Coleman *et al.*, 1994a. ¶AH 6809 is also a prostanoid DP receptor antagonist (see Fig 1.2).

3.1.2 Prostanoid receptors on human neutrophils

Numerous publications have documented the anti-inflammatory effect of the naturally occurring prostaglandins; PGE_1, PGE_2 and PGD_2 on isolated human neutrophils, such as inhibition of degranulation, superoxide generation, adhesion and injury to endothelial cells, (Bourne *et al.*, 1971, Lad *et al.*, 1985, Chopra & Webster, 1988, Hecker *et al.*, 1990). There have been conflicting reports of the ability of prostacyclin (PGI_2) and the IP agonist, iloprost, to inhibit human neutrophil activation (Boxer *et al.*, 1980, Gryglewski *et al.*, 1987, Riva *et al.*, 1990, Hecker *et al.*, 1990). The evidence, however, suggests that IP agonist inhibition of human neutrophil adhesion to endothelial cells may be due to an effect on the endothelial cell rather than the neutrophil (Boxer *et al.*, 1980 and Riva *et al.*, 1990). Weak inhibition of superoxide generation by PGI_2 and iloprost has only been observed by some groups; and then only at very high concentrations (Gryglewski *et al.*, 1987 and Hecker *et al.*, 1990) compared to the activity at other IP receptors such as on the human platelet (Dong *et al.*, 1986, Keery & Lumley, 1988).

PGE_1 and PGE_2 in common with other inhibitors of human neutrophil activation, such as β-adrenergic agonists (Nielson, 1987), also induced cAMP accumulation; and this has been proposed as a common mechanism of action. This is supported by the ability of dibutyryl cAMP (a cell-permeable analogue of cAMP) to inhibit neutrophil degranulation and superoxide generation (Bourne *et al.*, 1972).

Intravenous infusion of PGE_1 into humans also inhibits fMLP-stimulated lysosomal enzyme release from neutrophils *ex vivo* (Fantone *et al.*, 1981) demonstrating a direct anti-inflammatory effect of systemic prostaglandins on human neutrophils. This supports the observations made in animal models of the anti-inflammatory effects of stable prostaglandins (Rampart & Williams, 1986).

However, there has been little effort to pharmacologically characterize these human neutrophil prostanoid receptors. The generally accepted view, that the human neutrophil EP receptors are of the EP_2 subtype is based largely on circumstantial evidence, for instance PGE_1 and PGE_2 inhibit neutrophil activation and increase cAMP levels, and EP_2 receptors are positively linked to adenylate cyclase (Coleman *et al.*, 1990 and Yeardley *et al.*, 1993).

Few binding studies of human neutrophil prostanoid receptors have been reported. There is only one detailed binding study on PGD_2 and PGE_2 receptors in human neutrophils (Rossi & O'Flaherty, 1989). PGD_2 and PGE_2 bound to distinct sites; and described the presence of two binding sites for PGE_2 and one for PGD_2. For PGE_2, there was a high affinity site (K_d=10^{-9}M; 150 specific binding sites/cell) and a low affinity site (K_d=10^{-7}M; 5800 specific binding sites/cell), whilst there were 5100 binding sites for PGD_2 with a K_d of $1.3x10^{-8}$M. The authors suggested that the measured high affinity K_d values correlated with the inhibitory concentrations of the prostaglandins. However, this is unlikely to be the case for PGE_2 as with the majority of tissues the agonist occupancy curve is positioned to the right of functional E/[A] curve. Since the vast amount of literature on PGE_2 inhibition of human neutrophil activation, including the authors' own PGE_2-mediated inhibition of enzyme release, the position of E/[A] curve for PGE_2 (the $p[A_{50}]$) is to the right of the high affinity K_d, and therefore does not correlate with a binding constant K_d of 10^{-9}M. The K_d of 10^{-7}M for the lower affinity site for PGE_2 binding may be more consistent with the functional PGE_2 data, and the higher affinity site may represent either a higher affinity state of the same receptor or another EP receptor. No synthetic prostanoid antagonists or selective agonists were used to displace the labelled PGD_2 or PGE_2 binding, only unlabelled PGD_2 and PGE_2.

3.1.3 Aims

The objectives of this chapter were:-

1. To conduct a rigorous pharmacological characterization of the inhibitory prostanoid receptors present on human neutrophils, using inhibition of fMLP-stimulated superoxide generation as the functional assay.

2. To establish the relationship between prostanoid EP receptor agonist mediated inhibition of fMLP-stimulated neutrophil superoxide generation and cAMP elevation.

3.2 RESULTS

3.2.1 Effect of prostanoid EP agonists on superoxide generation by human neutrophils

There was little superoxide (O_2^-) generation from unstimulated human neutrophils (less than 0.1 nmol O_2^- 10^6 neutrophils^{-1}); but fMLP (10^{-7}M)-stimulated neutrophils generated 9.9±0.5 nmol O_2^- 10^6 neutrophils^{-1} (n=30). PGE_2 and PGE_1 inhibited fMLP (10^{-7}M)-stimulated superoxide generation with similar potency; $p[A_{50}]$ values were 7.2±0.1 (n=30) and 6.8±0.1 (n=4) respectively, and achieved the same maximum response (Fig 3.1). The EP_2 selective agonists AH13205, 11-deoxy PGE_1, butaprost and viprostol were also inhibitors, as were the other agonists with described activity at EP_2 receptors; 16,16-dimethyl PGE_2 (EP_1>EP_2) and misoprostol (EP_2/EP_3 selective) -see Table 3.2. The concentration-effect curves of these agonists as inhibitors of superoxide generation are shown in Fig 3.1 and Table 3.2.

EP_1 and EP_3 selective agonists were weak inhibitors of fMLP-stimulated superoxide generation compared with their activity at EP_1 and EP_3 receptors (Fig 3.1). Sulprostone (EP_1/EP_3 selective), M&B 28,767 and GR 63799X (EP_3 selective agonists) (Lawrence *et al.*, 1992, Bunce *et al.*, 1990 respectively) failed to reach above 50% inhibition at the highest concentration tested (Table 3.2). No concentration-effect (E/[A]) curves could be generated for these agonists.

The metabolites and breakdown products of the PGE compounds were also active although less potent than the parent compounds, PGE_1 and PGE_2 (Table 3.2). The most potent were PGE_0 (13,14-dihydro-PGE_1), PGA_1 and PGA_2 followed by PGB_1 and PGB_2; whilst 13,14-dihydro-15-keto-PGE_2 was virtually inactive.

3.2.2 Effect of prostanoid DP, IP and TP receptor agonists

The naturally occurring DP receptor agonist PGD_2 and the selective synthetic agonist BW 245C (Giles *et al.*, 1989) also inhibited superoxide generation with $p[A_{50}]$ values of 7.8±0.1 (n=7) and 8.4±0.2 (n=6) as shown in Fig 3.2. The potent IP receptor agonists cicaprost and iloprost (Dong *et al.*, 1986) were weak inhibitors of fMLP-stimulated superoxide generation compared with their potency at IP receptors. The maximum effect of cicaprost was ~30% at 10^{-5}M (n=3) and $p[A_{50}]$ of iloprost was <5.0 (n=3). The E/[A] curves are shown in Fig 3.2. The TP agonist U46619 had little effect when tested up to 10^{-6}M (n=2).

Agonist		$-\log_{10}$ [A_{50}]M	Max % I	n
PGE_2		7.2±0.1	87±8	30
PGE_1		6.8±0.1	83±7	4
11-Deoxy PGE_1	(EP_2)	6.1±0.2	96±2	7
AH13205	(EP_2)	5.5±0.2	95±2	7
Butaprost	(EP_2)	5.0±0.3	68±22	3
Viprostol	(EP_2)	8.2±0.3	76±2	4
17-Phenyl-trinor PGE_2	(EP_1>EP_2)	4.9±0.1	61±6	7
16,16-Dimethyl PGE_2	(EP_1/EP_3)	5.9±0.3	73±5	4
Sulprostone	(EP_1/EP_3)	<4.0	15±5 (10^{-4}M)	3
M&B 28,767	(EP_3>EP_2)	4.6±0.1	39±2	3
GR 63799X	(EP_3)	<4.5	18-45 (3×10^{-5}M)	2
Misoprostol	(EP_2/EP_3)	6.3±0.4	78±5	3
PGA_1		6.3±0.2	90±3	3
PGA_2		5.8±0.2	96±3	3
PGB_1		5.1±0.2	76±14	4
PGB_2		5.2±0.2	73±11	4
PGE_0 (13,14-dihydro PGE_1)		6.2±0.2	84±4	3
15-keto-13,14-dihydro PGE_2		<4.8	19-64 (10^{-4}M)	2

Table 3.2 Comparison of the potency of prostanoid EP agonists as inhibitors of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Human neutrophils (10^6 ml^{-1}) were preincubated with the agonists for 5 min (37°C) in the presence of cytochalasin B (5μg ml^{-1}) prior to stimulation with fMLP (5 min, 37°C). Results shown are the mean±s.e.m p[A_{50}] ($-\log_{10}$ [A_{50}]M) and maximum inhibition achieved (% I) of the number of experiments indicated for each agonist performed in duplicate.

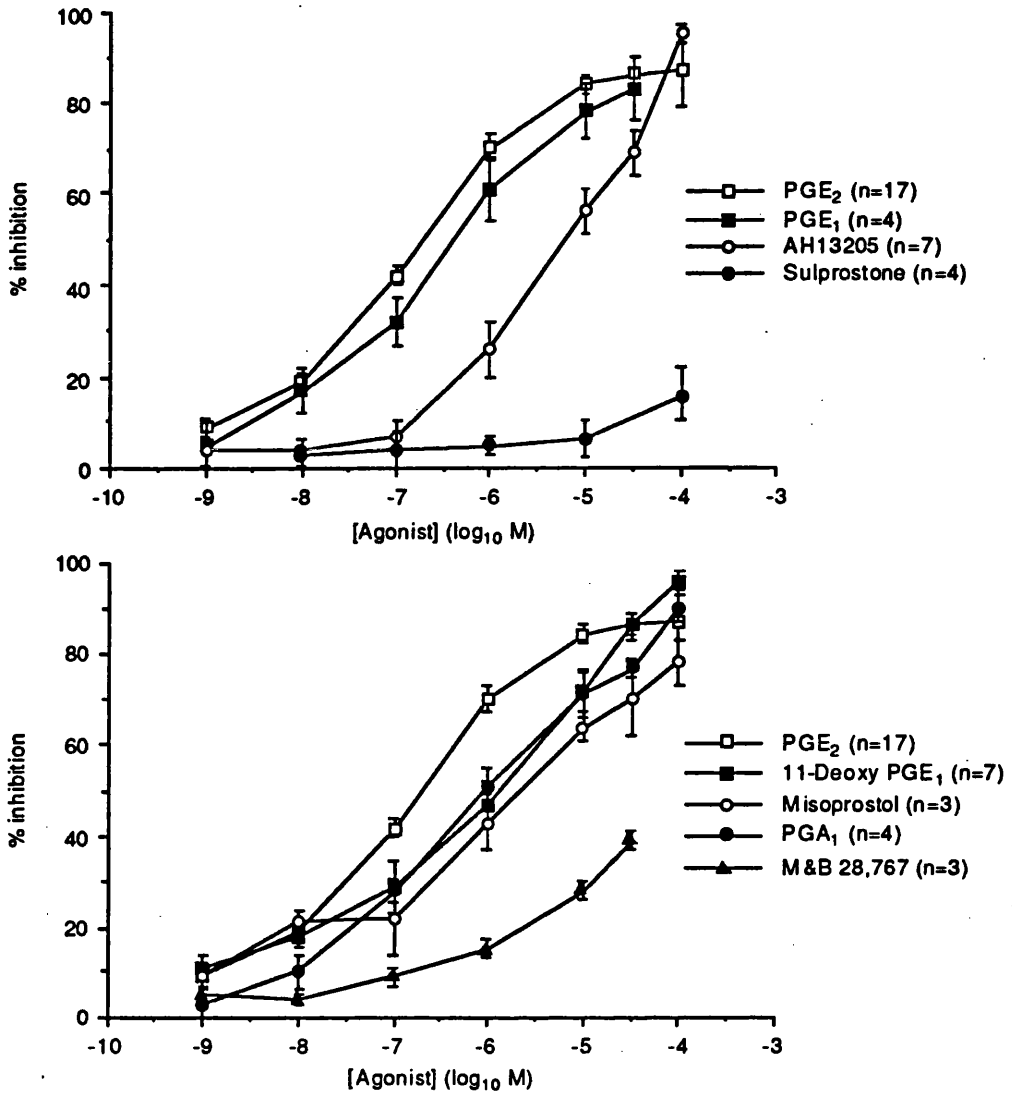


Fig 3.1 Effect of PGE_2, PGE_1 and prostanoid EP receptor agonists on fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with EP receptor agonists and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP for 5 min (37°C). The same PGE_2 data is shown in both panels for comparison. Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response for each agonist of the number of experiments indicated in the figure legends performed in duplicate.

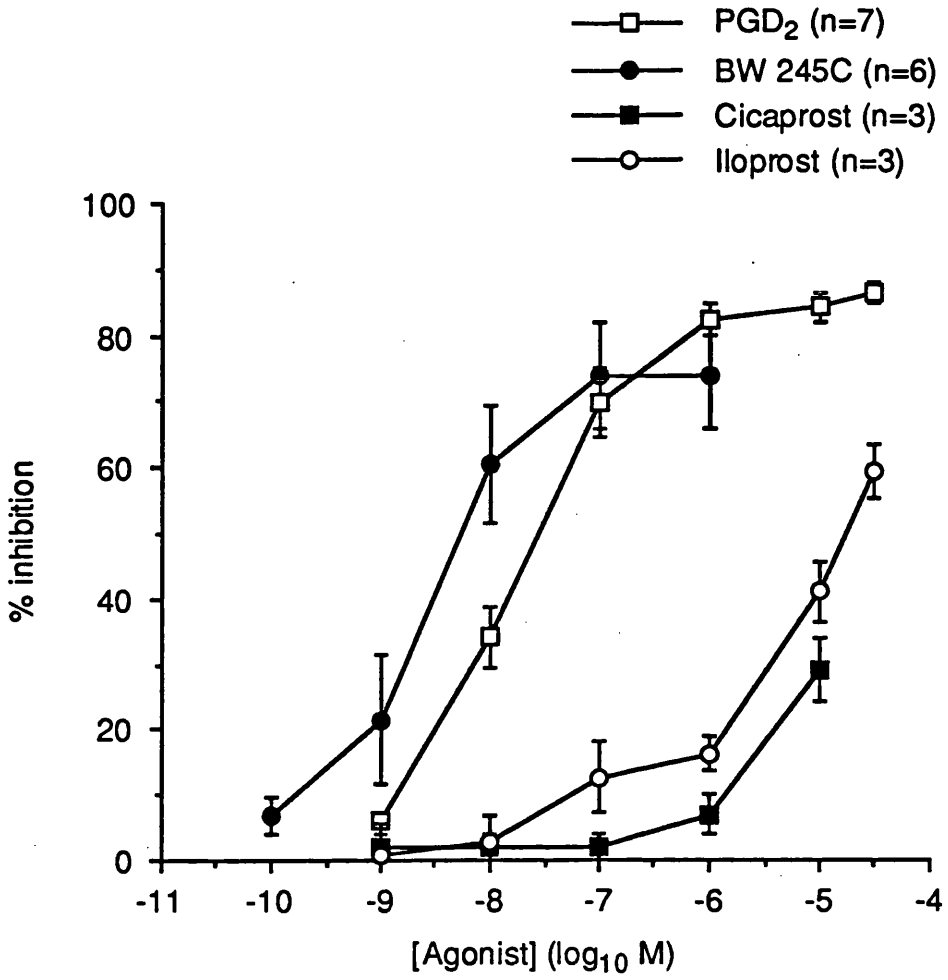


Fig 3.2 Effect of PGD_2, selective DP receptor agonist, BW 245C, and selective IP receptor agonists, cicaprost and iloprost, on fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with DP or IP receptor agonists in the presence of cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response for each agonist of the number of experiments indicated in the figure legends performed in duplicate.

3.2.3 Effect of prostanoid DP and EP receptor antagonists on PGE_2

The EP_1 selective antagonist SC 19220 (pA_2 5.4) (10^{-5}-10^{-4}M) did not antagonise the PGE_2 E/[A] curve (n=3). There was a slight nonsignificant leftward shift of the PGE_2 curve in the presence of 10^{-4}M SC 19220 (Fig 3.3).

AH 6809, the other EP_1 antagonist (pA_2 of 6.8 - Coleman *et al.*, 1987), produced a concentration-dependent (10^{-7}M - $3x10^{-6}$M) rightward shift of the PGE_2 E/[A] curve (Fig 3.4). There was no statistical difference in the goodness-of-fit (residual sum of squares) of the unconstrained Schild analysis compared with the analysis performed constraining the slope to unity, $F_{1,3}$=0.119, (P>0.05, degrees of freedom=1) (Leff & Giles, 1992). The pK_B value estimated using a modified Schild analysis, was 7.1±0.1 (data from n=5 donors) with the slope was constrained to unity.

This pK_B value was calculated from the earliest experiments conducted with AH 6809 on PGE_2-inhibition of O_2^- generation. Subsequent experiments using 10^{-5}M AH 6809 performed over the duration of this entire study (5 years) using a wider range of blood donors showed that the degree of antagonism observed was extremely variable and was consistently lower than that predicted from the pK_B value of 7.1 (Fig 3.5). A more accurate estimate of a pA_2 value may actually be closer to 6.0 than 7.1 as the concentration-ratio (CR) was 1.3±0.1$\log_{10}$ units (n=68). However in some donors, the degree of antagonism observed using 10^{-5}M AH 6809 was similar to $3x10^{-6}$M, suggesting that a 'wall' existed limiting the amount of antagonism obtainable. Such a situation may arise if more than one receptor for PGE_2 coexists in the neutrophil mediating inhibition of fMLP-stimulated superoxide generation, and only one of which is antagonised by AH 6809. Variability of the relative expression of these AH 6809-sensitive and insensitive prostanoid EP receptors between individuals may explain the diversity of AH 6809 antagonism in the different

neutrophil preparations.

AH 6809, up to 10^{-5}M, and under these experimental conditions did not stimulate O_2^- generation. However, fMLP-stimulated O_2^- generation was increased in the presence of AH 6809 (10^{-5}M) to 147±6% of the control fMLP response (n=68). This apparent enhancement was variable and had no correlation with the degree of antagonism of PGE_2 observed in each experiment (Fig 3.5, n=68).

As AH 6809 is also a DP receptor antagonist, another DP antagonist was evaluated to determine whether the inhibitory effect of PGE_2 was mediated at the DP receptor. The selective and potent DP receptor antagonist BW A868C (pA_2 9.3, Giles *et al.*, 1989) was without effect on the PGE_2 E/[A] curve (n=5) at 10^{-7}M, a concentration which caused a 2.4±0.3 $\log_{10}$ unit rightward shift of the PGD_2 E/[A] curve (n=5) (Fig 3.6). BW A868C (10^{-7}M) also had no effect on the antagonism of the PGE_2 E/[A] curve by 10^{-5}M AH 6809 (n=3) (Fig 3.7). The weak EP_4 receptor antagonist AH23848B (pA_2 4.9-5.4, Coleman *et al.*, 1994a) depressed the control fMLP superoxide response at $3x10^{-5}$M by 77.9±4.0% (n=3), but not at 10^{-5}M (106±18% of control fMLP response, n=3). AH23848B (10^{-5}M) had no effect on the PGE_2 E/[A] curve (n=3) (Fig 3.8). The control PGE_2 $p[A_{50}]$ was 7.5±0.2 (maximum inhibition of 86.3±1.8%, n=3) compared to 7.4±0.1 (maximum inhibition of 80.7±3.5%, n=3) in the presence of AH23848B (10^{-5}M). AH23848B is more potent as a TP receptor antagonist (Brittain *et al.*, 1984) but the structurally related compound GR 32191B, also a TP receptor antagonist (pA_2 8.2-8.8 in human platelets, Lumley *et al.*, 1989), had no effect on fMLP stimulated superoxide generation itself ($3x10^{-6}$M, n=2) and did not affect the PGE_2 inhibition curve ($3x10^{-6}$M, n=2).

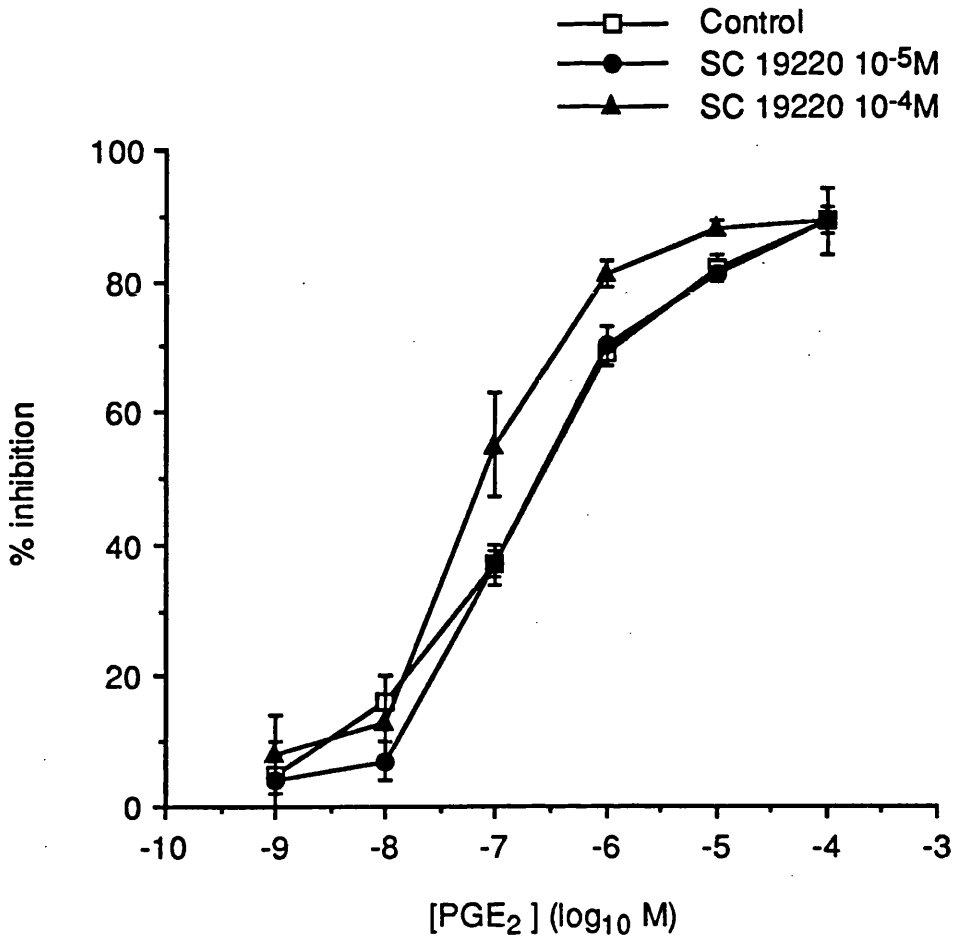


Fig 3.3 Effect of prostanoid EP_1 receptor antagonist, SC 19220 (10^{-5}M and 10^{-4}M) on PGE_2-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with SC 19220 (60 min, room temperature) prior to addition of PGE_2 and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 3 separate experiments performed in duplicate.

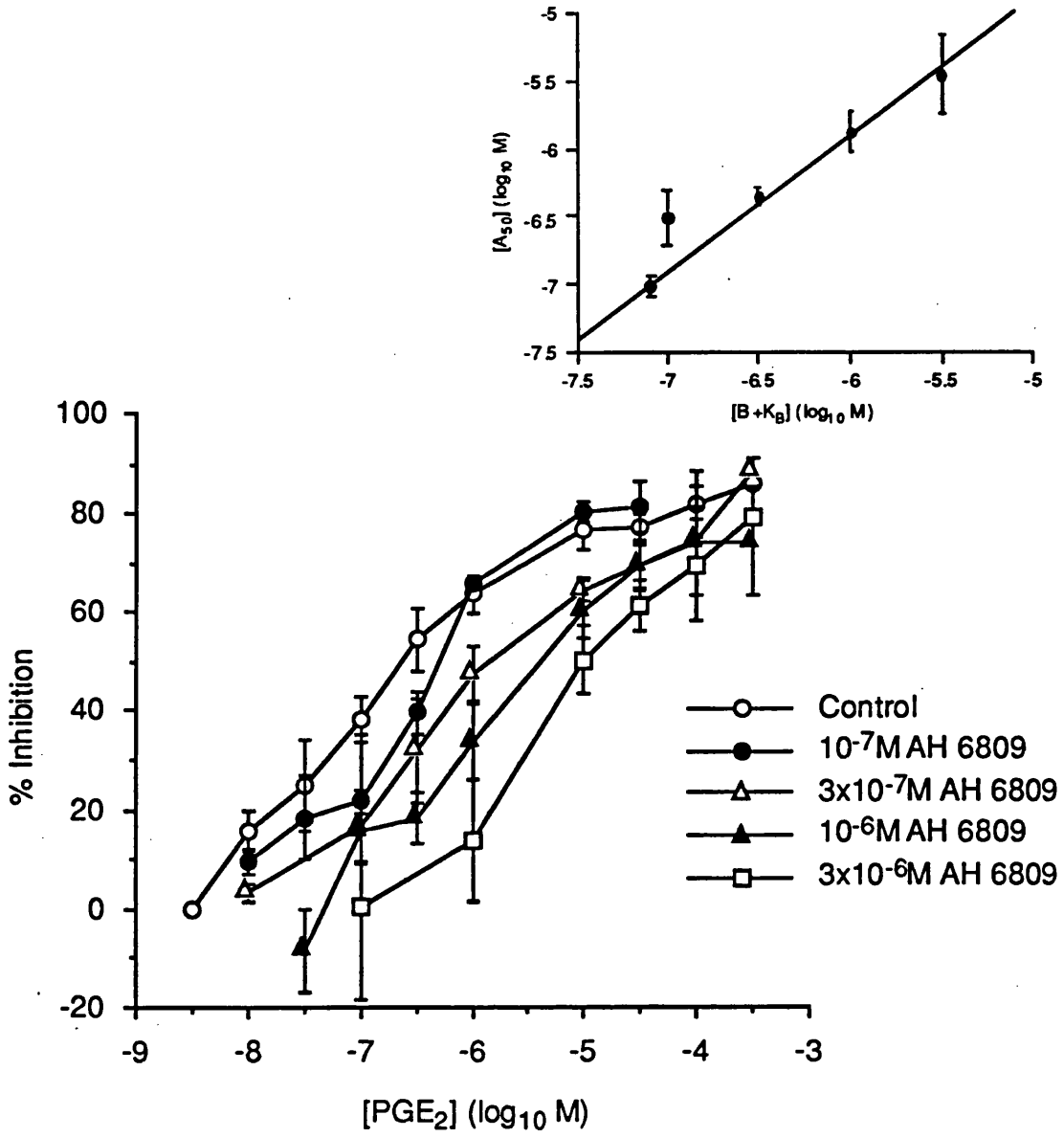


Fig 3.4 Effect of prostanoid DP/EP$_1$ receptor antagonist AH 6809 (10^{-7}-3×10^{-6}M) on PGE$_2$-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with AH 6809 (60 min, room temperature) prior to addition of PGE$_2$ and cytochalasin B (5µg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 5 separate experiments performed in duplicate. Inset shows the corresponding modified Schild plot as described in the text, where A and B represent the agonist (PGE$_2$) and antagonist (AH 6809) respectively.

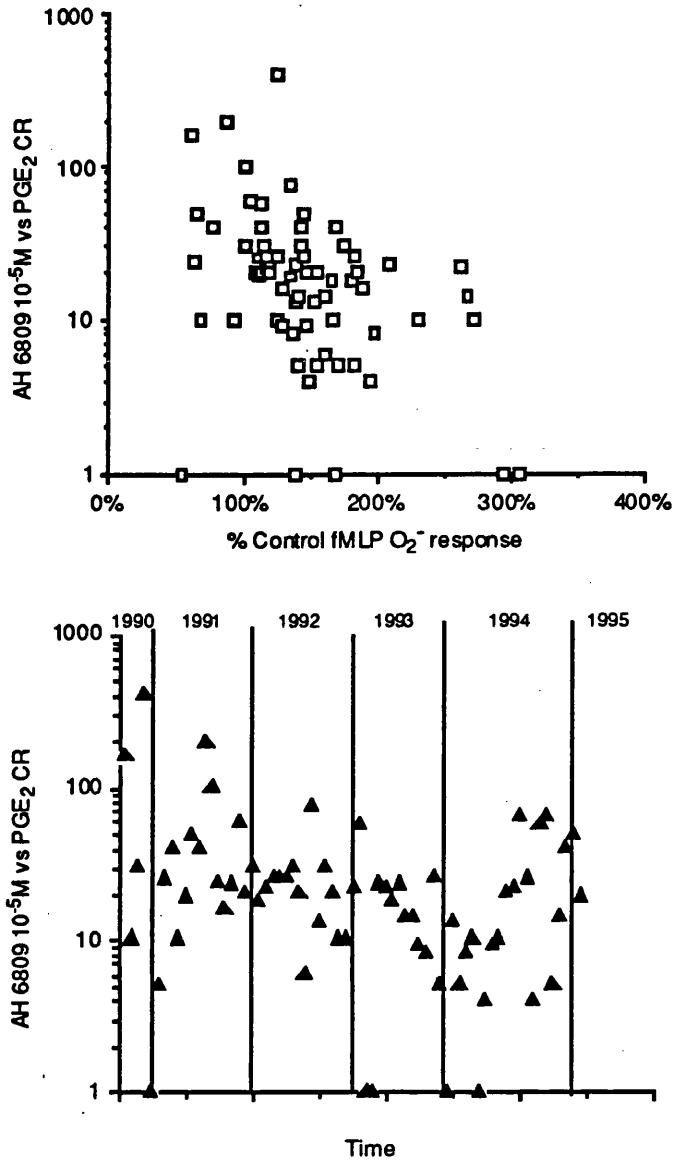


Fig 3.5 Variability of AH 6809 (10^{-5}M) antagonism of PGE_2-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils (upper panel), and lack of correlation between AH 6809 antagonism and enhancement of fMLP (10^{-7}M)-stimulated superoxide generation (lower panel). Results show AH 6809-potentiation of fMLP-stimulated superoxide generation (control fMLP response=100%) plotted against AH 6809 (10^{-5}M) antagonism of PGE_2 expressed as the concentration ratio (CR) estimated in the same experiments (n=68 experiments).

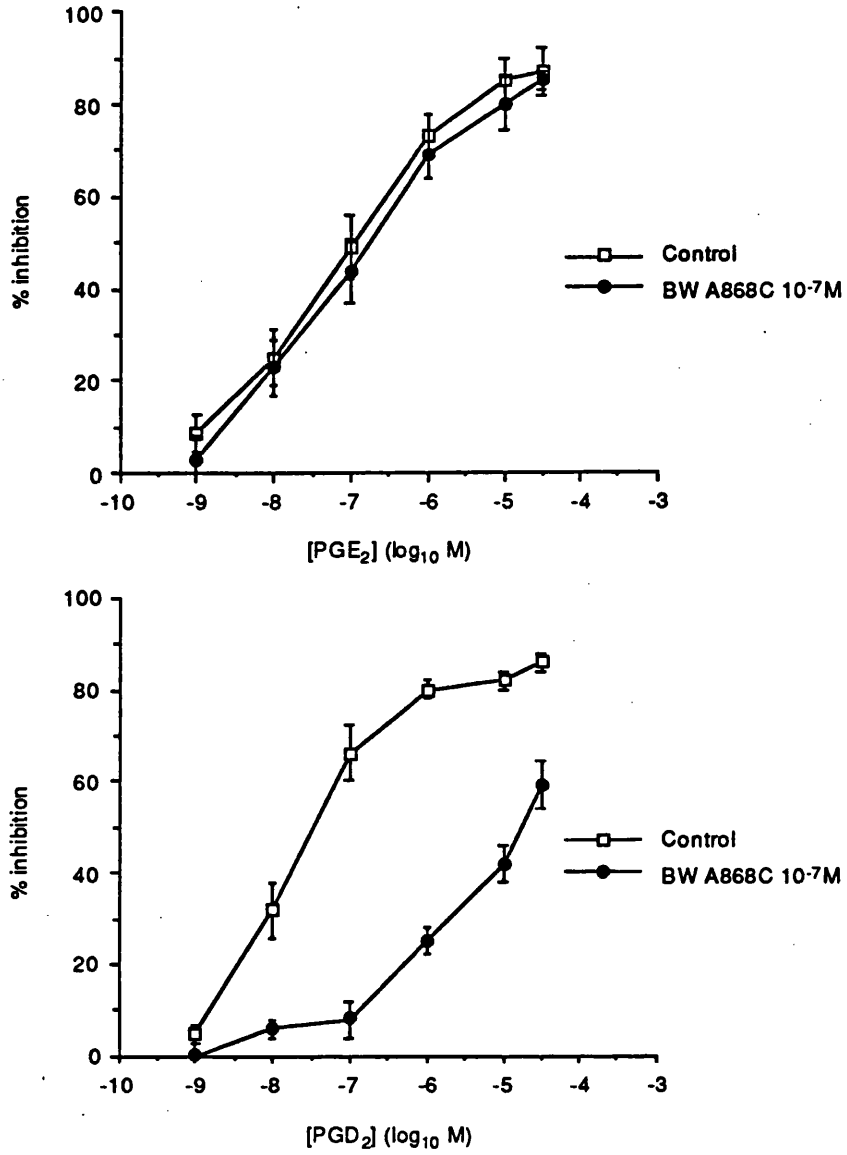


Fig 3.6 Effect of prostanoid DP receptor antagonist BW A868C (10^{-7}M) on PGE_2 (upper panel) and PGD_2 (lower panel)-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with BW A868C (60 min, room temperature) prior to addition of prostaglandins and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 5 separate experiments performed in duplicate.

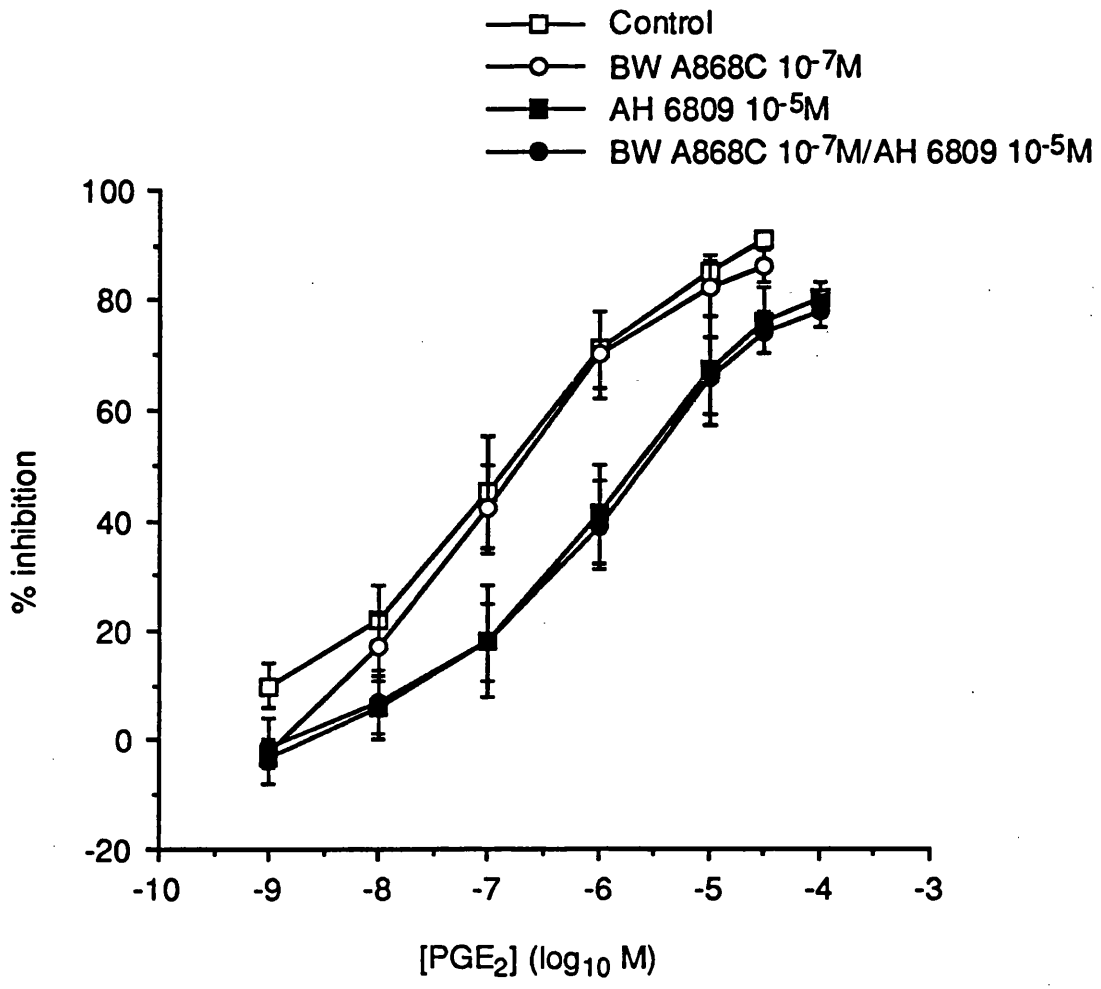


Fig 3.7 Effect of prostanoid DP receptor antagonist BW A868C (10^{-7}M) on AH 6809 (10^{-5}M) antagonism of PGE_2-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with BW A868C+/-AH 6809 (60 min, room temperature) prior to addition of PGE_2 and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 3 separate experiments performed in duplicate.

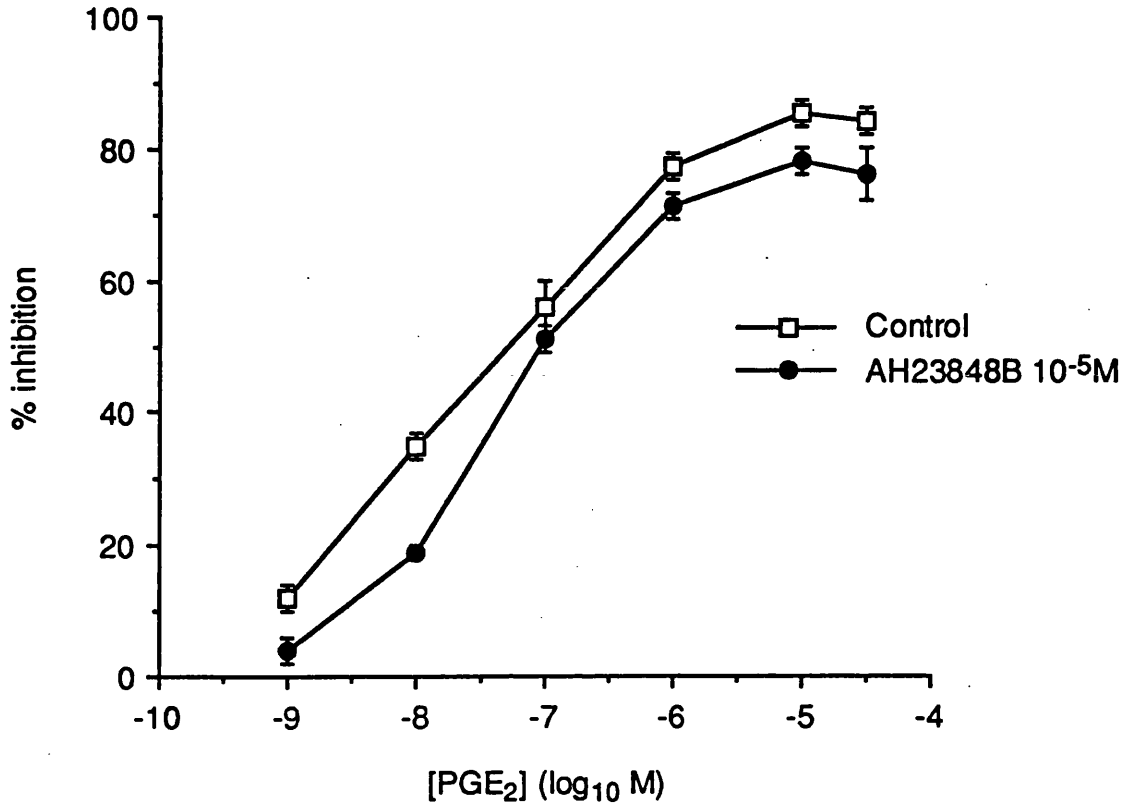


Fig 3.8 Effect of prostanoid EP_4 receptor antagonist AH23848B (10^{-5}M) on PGE_2-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with AH23848B (60 min, room temperature) prior to addition of prostaglandins and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 3 separate experiments performed in duplicate.

3.2.4 Specificity and selectivity of AH 6809 antagonism

The adenosine A_2 receptor agonist, NECA, and the direct activator of adenylate cyclase, forskolin, both inhibited fMLP stimulated superoxide generation. The $p[A_{50}]$ value for NECA was 7.9±0.1 and the pIC_{50} was <4.5 for forskolin ($p[A_{50}]$ was not calculated as no clear maximum was reached at $3x10^{-5}M$). The maximum inhibition reached by NECA and forskolin were 85.3±3.5% and 43.0±6.0% respectively (at $3x10^{-5}M$, n=3 for both). AH 6809 at $10^{-5}M$ had no effect on the E/[A] curves of NECA ($p[A_{50}]$=7.9±0.1, maximum inhibition of 77.3±1.0%) or forskolin (pIC_{50} <4.5, maximum inhibition of 37.0±3.0%) shown in Fig 3.9 (n=3 for both). This concentration of AH 6809 did however cause a rightward shift of the E/[A] curve of the EP_2 selective agonist AH13205, although the shift was not parallel (Fig 3.10). An approximately 20 fold parallel rightward shift of the lower part of the AH13205 curve was observed at $10^{-5}M$ AH 6809 (n=3), but at AH13205 concentrations of $10^{-5}M$ and higher, less antagonism was observed.

AH 6809 under the same conditions antagonised BW 245C inhibition of fMLP-stimulated superoxide generation (Fig 3.11) with a pA_2 value of 6.6±0.1 (slope=1.0±0.1, n=3 experiments). The selective prostanoid DP receptor antagonist BW A868C ($10^{-7}M$) also antagonised BW245C, by displacing the E/[A] curve by 2.1±0.5 $\log_{10}$ units (n=3) (Fig 3.11).

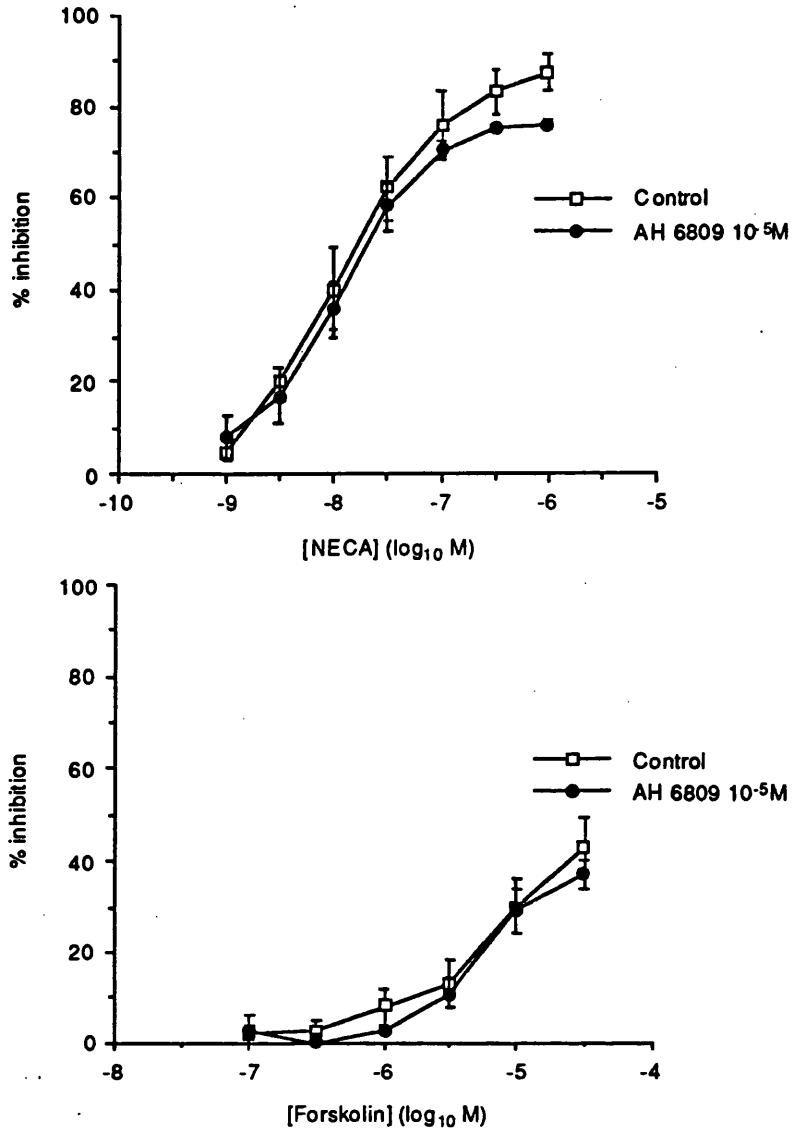


Fig 3.9 Effect of AH 6809 (10^{-5}M) on adenosine receptor agonist NECA (upper panel) and direct adenylate cyclase stimulator forskolin (lower panel)-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with AH 6809 (60 min, room temperature) prior to addition of inhibitors and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 3 separate experiments performed in duplicate.

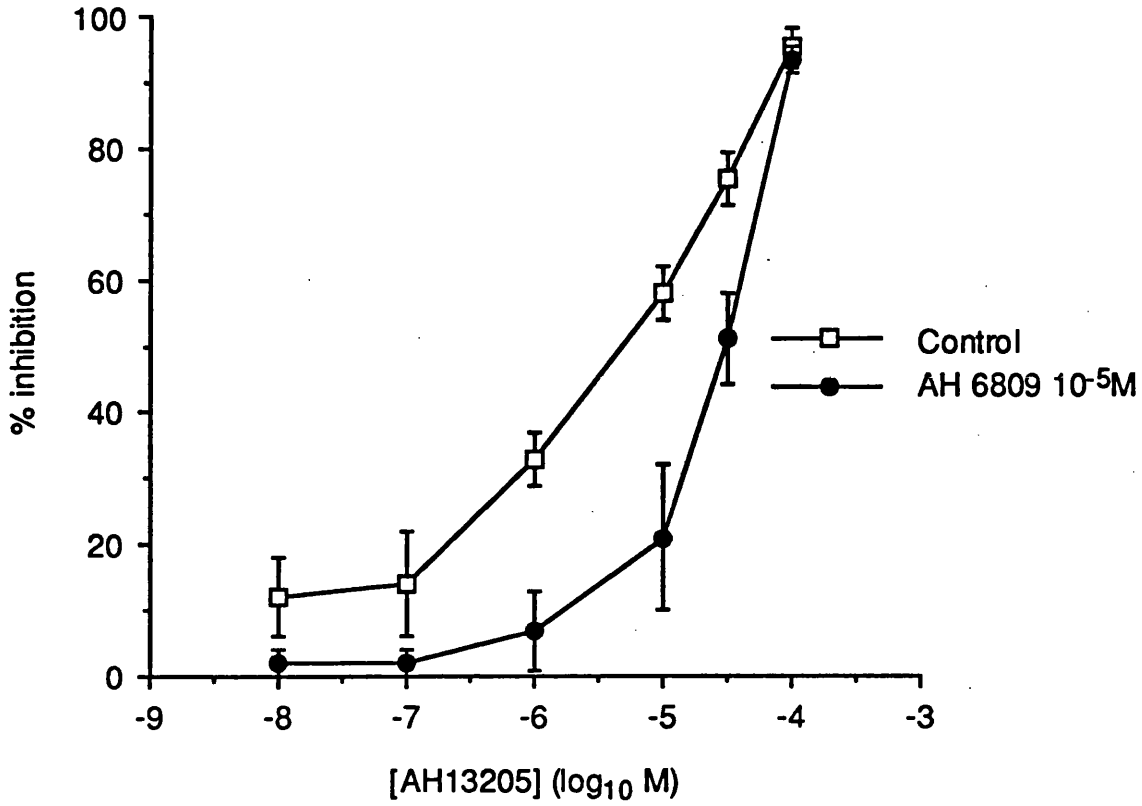


Fig 3.10 Effect of AH 6809 (10^{-5}M) on selective EP_2 receptor agonist AH13205-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with AH 6809 (60 min, room temperature) prior to addition of AH13205 and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 3 separate experiments performed in duplicate.

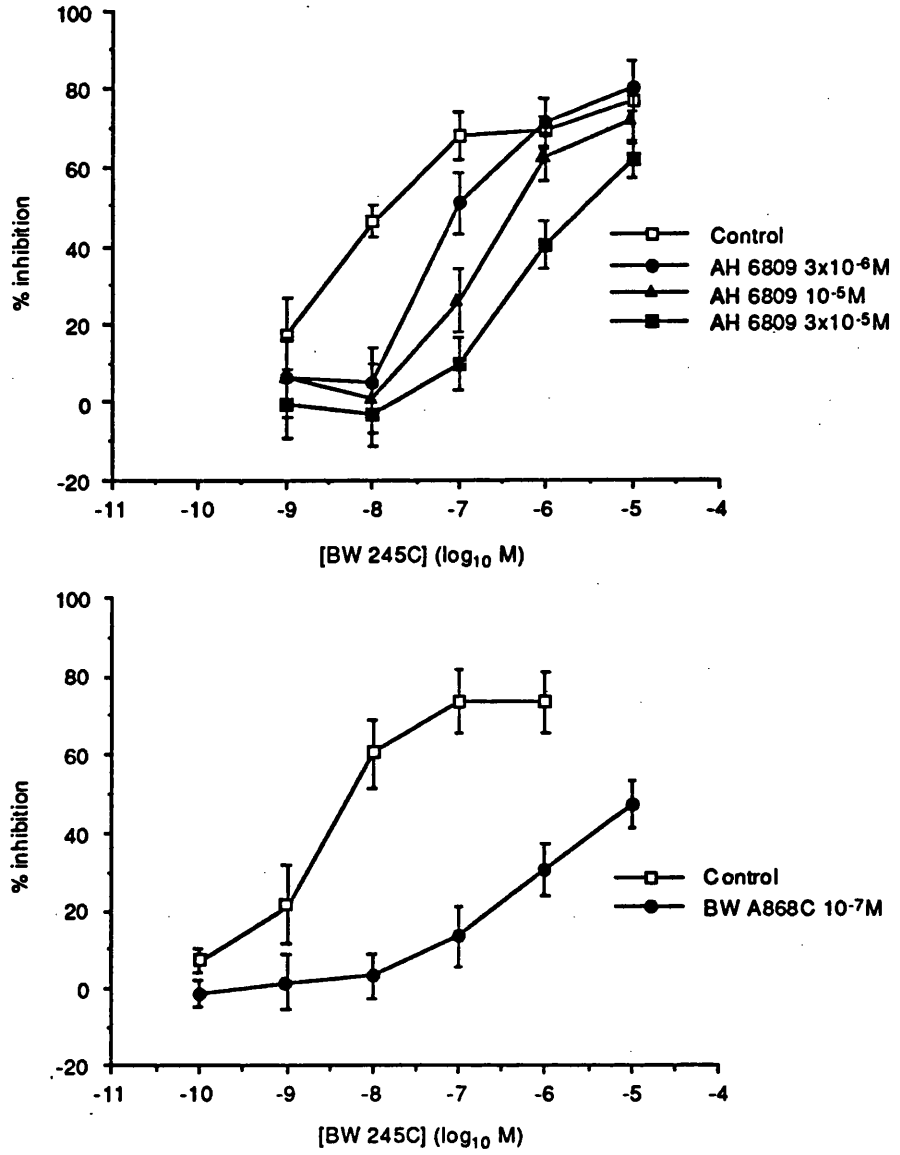


Fig 3.11 Effect of AH 6809 ($3x10^{-6}M$-$3x10^{-5}M$) (upper panel) and BW A868C ($10^{-7}M$) (lower panel) on BW 245C-mediated inhibition of fMLP ($10^{-7}M$)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with AH 6809 or BW A868C (60 min, room temperature) prior to addition of BW 245C and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) and then stimulated with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of fMLP-stimulated superoxide response of 3 (AH 6809) and 4 (BW A868C) separate experiments performed in duplicate.

3.2.5 Effect of PGE_2 and EP agonists on cAMP accumulation in human neutrophils

PGE_2 stimulated increases in cAMP accumulation in human neutrophils in the presence of $5x10^{-4}M$ IBMX (Fig 3.12), by 7.5±3.0 pmol cAMP 10^6 neutrophils^{-1} (n=21) above basal levels of 1.2±0.2pmol cAMP 10^6 neutrophils^{-1} (n=21) with a $p[A_{50}]$ of 6.8±0.1 (n=21). Other EP receptor agonists 11-deoxy PGE_1, misoprostol, AH13205 and also PGA_1 also increased cAMP accumulation (Fig 3.12). In individual experiments 11-deoxy PGE_1 and misoprostol were full agonists compared to PGE_2 with a maximum response, α=1. The $p[A_{50}]$ values for 11-deoxy PGE_1 and misoprostol were 5.6±0.2 and 5.2±0.2 respectively (n=4 for both). However PGA_1 ($p[A_{50}]$=6.3±0.2) and AH13205 ($p[A_{50}]$=5.8±0.1) were found to be partial agonists with α values of 0.62±0.04 and 0.34±0.03 respectively compared with PGE_2 response, α=1, which were significantly lower than PGE_2, $p<0.05$ for both (n=3).

3.2.6 Application of the Operational Model to EP agonist data in superoxide and cAMP assays

The experimental potency orders (using $p[A_{50}]$ values), of the prostanoid EP agonists PGE_2, PGA_1, AH13205, misoprostol and 11-deoxy-PGE_1 in the fMLP-stimulated superoxide assay and the cAMP assay were different (Table 3.3). However, the analysis was complicated by the partial agonist activity of AH13205 and PGA_1 compared with PGE_2 in the cAMP assay.

After application of the Operational model to the same experimental data in both superoxide and cAMP assays to simulate an increase in receptor reserve; all the agonists reached the same maximum response and the potency orders in the 2 assays was similar. The latter suggests that prostanoid EP agonists inhibited fMLP-stimulated superoxide generation and

stimulated cAMP elevation by activating the same receptor.

3.2.7 The effect of AH 6809 and AH23848B on PGE_2 stimulated cAMP accumulation by human neutrophils

AH 6809 (10^{-5}M) had no effect on basal cAMP levels compared to control neutrophils which were 0.7±0.1 and 0.8±0.1 pmol 10^6 neutrophils^{-1} respectively (n=11). AH 6809 (10^{-5}M) did however antagonise PGE_2 stimulated cAMP accumulation by human neutrophils (Fig 3.13), the rightward shift was 1.3±0.1 $\log_{10}$ units n=11 which approximated to a pA_2 value of 6.3. This was consistent with the observed antagonist activity of AH 6809 (at 10^{-5}M) against PGE_2 inhibition of fMLP-stimulated superoxide generation by human neutrophils.

AH23848B ($3x10^{-5}$M) had no effect on basal cAMP levels compared to human neutrophils which were 0.8±0.2 and 1.0±0.1 pmol 10^6 neutrophils^{-1} respectively (n=3). Neither did AH23848B ($3x10^{-5}$M) produce a rightward shift of the PGE_2 cAMP stimulation curve (Fig 3.13), the DR was 0.1±0.1 $\log_{10}$ units (n=3), indicating that AH23848B did not antagonise PGE_2-mediated cAMP elevation in human neutrophils.

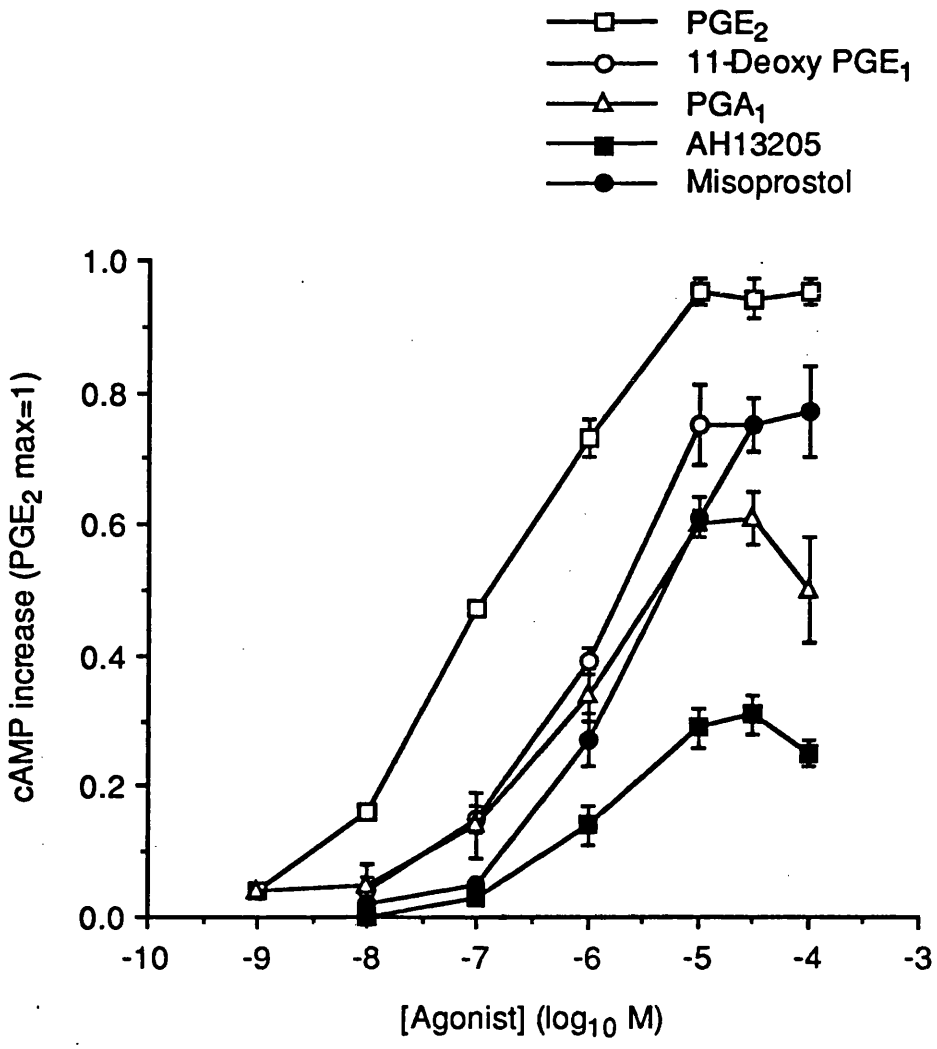


Fig 3.12 Effect of PGE_2 and prostanoid EP receptor agonists on cAMP levels in human neutrophils. Neutrophils ($3x10^6$ per determination) were preincubated with IBMX ($5x10^{-4}$M, 5 min, 37°C) prior to stimulation with prostanoid agonists (5 min, 37°C). Results shown are the mean±s.e.m cAMP increase for each agonist normalised with respect to the PGE_2 maximum (=1) of 3-10 separate experiments performed in duplicate.

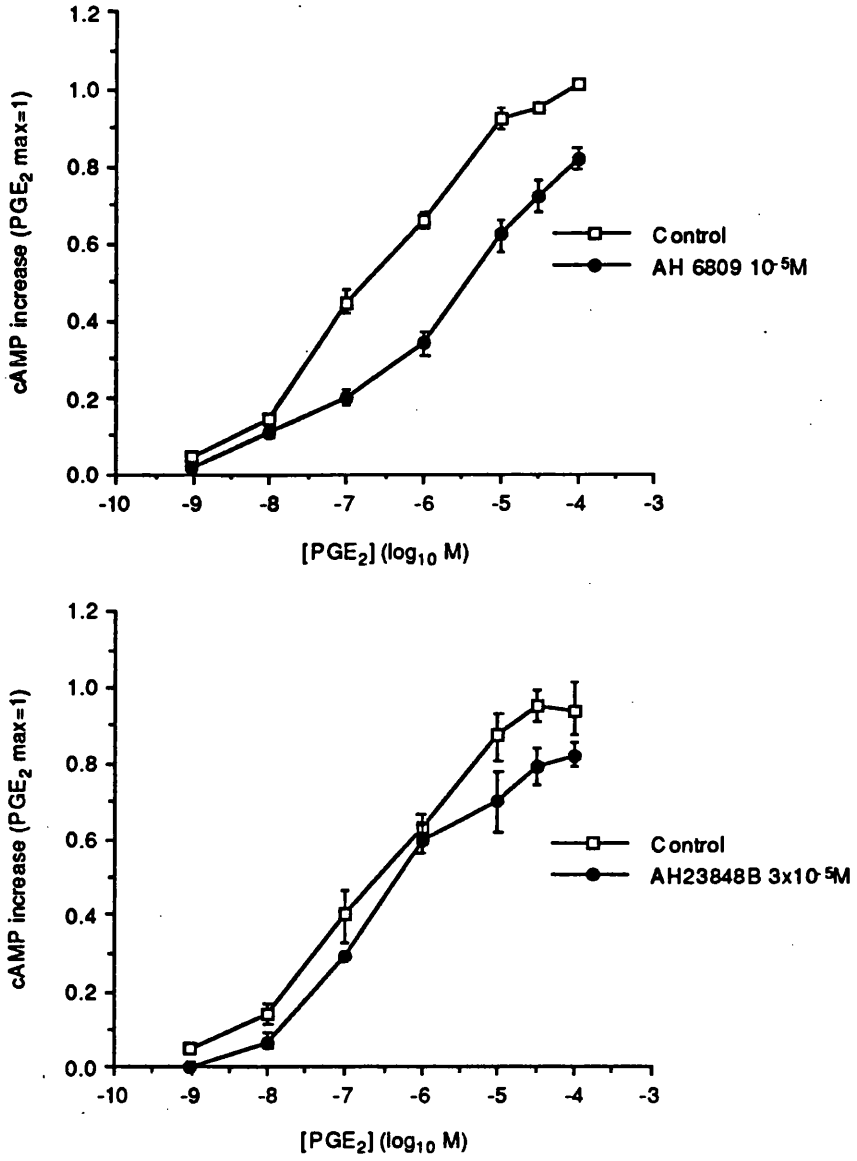


Fig 3.13 Effect of AH 6809 (10^{-5}M) (upper panel) and AH23848B ($3x10^{-5}$M) (lower panel) on PGE_2-stimulated cAMP accumulation by human neutrophils. Neutrophils ($2x10^7$ ml^{-1}) were preincubated with the antagonists for 3 min (room temperature) followed by the addition of IBMX ($5x10^{-4}$M, 5 min, 37°C) prior to stimulation with prostanoids (5 min, 37°C). Results shown are the mean±s.e.m cAMP increase normalised with respect to the control PGE_2 maximum (=1) of 11 (AH 6809) and 3 (AH23848B) separate experiments performed in duplicate.

	Experimental				
Superoxide	PGE_2>PGA_1≈misoprostol≈11-deoxy PGE_1>AH13205				
$p[A_{50}]$	7.2	6.3	6.3	6.1	5.5
cAMP	PGE_2>PGA_1>AH13205≈11-deoxy PGE_1 ≈misoprostol				
$p[A_{50}]$	6.8	6.3	5.8	5.6	5.2
α	1.0	0.62	0.34	1.0	1.0
	After Simulation				
Superoxide	PGE_2>PGA_1≈misoprostol≈11-deoxy PGE_1>AH13205				
$p[A_{50}]$	9.2	8.3	8.3	8.1	7.5
cAMP	PGE_2>PGA_1≈11-deoxy PGE_1≈misoprostol>AH13205				
$p[A_{50}]$	8.8	7.6	7.6	7.2	6.9
α	1.0	1.0	1.0	1.0	1.0

Table 3.3 Application of Operational Model of Agonism to EP agonist data in human neutrophil superoxide and cAMP assays. An increase in receptor reserve was simulated in the experimental data using the Operational Model in order to render all the agonists full for comparison of agonists potency orders.

3.3 DISCUSSION

The results presented in this chapter suggest that the prostanoid EP receptor mediating inhibition of fMLP stimulated superoxide generation by human neutrophils to be pharmacologically distinct from the prostanoid EP_2 receptor. Initial characterization showed that this inhibitory EP receptor exhibited an agonist potency order consistent with the EP_2 subtype. In addition to this, the same agonist potency order was obtained for cAMP accumulation in human neutrophils suggesting both functions were mediated by the same receptor. However, AH 6809 (an EP_1/DP receptor antagonist) antagonism of PGE_2 inhibition of fMLP-stimulated superoxide generation by the human neutrophil appears to differentiate it from the EP_2 receptor according to the existing classification.

3.3.1 Inhibitory prostanoid EP receptors on human neutrophils

In the present study, human neutrophils were shown to express prostanoid EP and DP receptors; activation of which inhibited O_2^- generation stimulated by fMLP. The presence of these inhibitory prostanoid receptors on human neutrophils is confirmed by others on the activity of PGE_1, PGE_2 and PGD_2 on O_2^- generation, LTB_4 release and degranulation by human neutrophils (Ney & Schrör, 1991; Wheeldon & Vardey, 1993). Inhibition of fMLP-stimulated O_2^- generation by the selective IP (cicaprost and iloprost) and TP (U46619) receptor agonists was only observed at high concentrations compared with those selective for their respective receptors (Dong *et al.*, 1986). This suggested that neither receptor was present on human neutrophils and is supported by the findings of others (Hecker *et al.*, 1990, Wheeldon & Vardey, 1993). Inhibitory effects of IP and TP receptor agonists on human neutrophil activation may therefore be mediated by interacting with DP and/or EP receptors.

The activity profile and potency order of the wide range of prostanoid EP agonists tested

suggest that the inhibitory receptor on the human neutrophil is of the EP_2 subtype. The range of compounds tested in this study is an expansion on the preliminary reported data (Li *et al.*, 1993) and more extensive than those more recently published in the literature (Wheeldon & Vardey, 1993, Armstrong & Talpain, 1994, Talpain *et al.*, 1994). Similar agonist potency orders have been reported for the more widely used agonists; AH13205, butaprost, 11-deoxy PGE_1, misoprostol and sulprostone as inhibitors of opsonised zymosan stimulated-LTB_4 generation (Wheeldon & Vardey, 1993) and fMLP-stimulated O_2^- formation (Armstrong & Talpain, 1994) by human neutrophils.

In addition, this study has established that the same agonist potency order exists in the human neutrophil for inhibition of O_2^- generation and stimulation of cAMP accumulation. This provides further evidence for the human neutrophil prostanoid EP receptor as being similar to the EP_2 subtype as the signal transduction pathway for the EP_2 receptor is cAMP (Coleman *et al.*, 1990 and Yeardley *et al.*, 1993). Additional quantitative information on agonist activity was obtained from the cAMP measurements, as some of the agonists were partial agonists in this second messenger assay, i.e. PGA_1 and AH13205. In contrast, all the prostanoid EP agonists including PGA_1 and AH13205 were apparently full agonists in the O_2^- functional assay. In other 'EP_2-receptor' containing preparations such as the rabbit ear artery; AH13205 is a full agonist (Nials *et al.*, 1993). However, in the human myometrium, PGE_2, butaprost and AH13205 stimulate cAMP elevation via an EP_2-receptor; but notably AH13205 is a partial agonist. Using the Operational Model (Leff *et al.*, 1990), the affinity and efficacy of the partial agonists could be estimated which further characterized and quantified the properties of the agonist at the neutrophil prostanoid EP receptor. Conclusive proof of these partial agonists AH13205 and PGA_1 acting at the same receptor as PGE_2 could only be provided by partial agonist-full agonist interaction experiments with PGA_1 or AH13205 with PGE_2 (full agonist). However, the poor potency of these partial agonists and

the precision of the cAMP assay do not permit such a study. In addition, the apparent partiality of PGA_1 and AH13205 may be explained by the model proposed by Szabadi (1977); in which an agonist can activate two functionally antagonistic receptor populations. However, the agonist potency orders obtained for the prostanoid EP agonists in the cAMP assay after simulation of increased receptor reserve do suggest that both PGA_1 and AH13205 are truly partial agonists.

The EP_1 selective antagonists SC 19220 and AH 6809 were tested to further confirm the EP_2 identity deduced from agonist potency information. As would have been expected from the classification, SC 19220 (up to 10^{-4}M) had no effect on PGE_2 inhibition of O_2^- generation, although this high antagonist concentration did appear to cause a leftward shift of the PGE_2 curve. Taking into account the weakness of SC 19220 as an EP_1 receptor antagonist (pA_2 5.4), the possibility of a non-specific effect cannot be excluded. Coleman *et al.*, (1987) also showed SC 19220 did not antagonise PGE_2 induced relaxation of the cat trachea, an EP_2 receptor containing tissue, up to a concentration of 10^{-4}M (pA_2 <4.0).

Coleman *et al.* (1987) also reported that AH 6809 had no antagonist activity at 10^{-5}M (pA_2 <5.0) in the cat trachea. These observations were the basis of the EP receptor antagonist profile of SC 19220 and AH 6809, and are upheld to the present time. However, few studies have actually used SC 19220 and AH 6809 to confirm agonist potency information on EP_2 receptors. The characterization of EP_2 receptors relied upon the activity of the EP_2 agonists butaprost and AY-23626 (EP_2/EP_3 selective, available in limited supply, but now exhausted), and inactivity of sulprostone (EP_1/EP_3 selective). Antagonists were only used to differentiate between EP_1 and EP_3 receptors as sulprostone was an agonist at both subtypes. Unexpectedly and contrary to the established classification, AH 6809 concentration-dependently caused a rightward shift of the PGE_2 neutrophil superoxide inhibition E/[A]

curve and the PGE_2 cAMP stimulation curve in the human neutrophil. This suggests that AH 6809 antagonises a response mediated via an EP_2 receptor.

The possibility of AH 6809 antagonism of PGE_2 being mediated at the DP receptor was excluded, as the more selective and potent DP receptor antagonist BW A868C (at 10^{-7}M) had no effect on the PGE_2 inhibition of O_2^- generation. The same concentration of BW A868C (10^{-7}M) produced over 100 fold rightward shift of the PGD_2 curve which was consistent with its pA_2 value of 9.3 at the DP receptor. Neither was the antagonism of PGE_2 by 10^{-5}M AH 6809 (a concentration which produced the maximum rightward shift of the PGE_2 curve of 20-30 fold) affected by the presence of BW A868C (10^{-7}M), further confirming that DP receptors did not contribute to the inhibitory effect of PGE_2 or its antagonism by AH 6809. AH 6809 under the same assay conditions, did however antagonise DP receptor mediated inhibition of fMLP-stimulated O_2^- generation, causing rightward shifts of the E/[A] curves of BW 245C. This was consistent with the degree of antagonism of BW245C observed by Wheeldon & Vardey (1993) also using fMLP-stimulated O_2^- generation in human neutrophils as the assay system.

Further confirmation of the human EP_2 receptor antagonistic activity of AH 6809 was the 20-fold rightward shift of the AH13205 curve by AH 6809 (10^{-5}M). The antagonism was restricted to the lower part of the AH13205 curve which may reflect on the lack of potency of AH13205 as a prostanoid EP agonist; since even the control AH13205 E/[A] curve steepened at concentrations of $\geq 10^{-5}$M. These results suggest that AH13205 had non-specific effects on neutrophil function, and/or perhaps had effects on another inhibitory receptor at $\geq 10^{-5}$M.

The specificity of the AH 6809 antagonism was confirmed by its lack of effect on another (a non-prostanoid) inhibitor of human neutrophil activation, the adenosine A_2 receptor agonist NECA (Cronstein *et al.*, 1988). Neither did AH 6809 antagonise forskolin (the direct

activator of adenylate cyclase, De Souza *et al.*, 1983) inhibition of fMLP stimulated superoxide generation. Thus, AH 6809 was not acting indiscriminately and generally antagonising inhibitors of neutrophil activation; nor was it acting intracellularly post-activation of adenylate cyclase. These data support the specificity of AH 6809 as a prostanoid receptor antagonist, as it does not appear to interfere with the non-prostanoid receptors or the other components downstream of adenylate cyclase involved in inhibition of fMLP-stimulated O_2^- generation. AH 6809 (10^{-5}M) also antagonised PGE_2-stimulated cAMP accumulation by 1.3 $\log_{10}$ units i.e. similar to the degree of antagonism observed in the O_2^- assay, thereby providing yet more evidence for AH 6809 acting selectively at the prostanoid EP receptor.

Prostanoid receptor-selective antagonism by AH 6809 was not however observed by Talpain *et al.* (1994). They observed AH 6809 (10^{-5}M) antagonism of NECA inhibition of fMLP-stimulated O_2^- generation which was equal to if not greater than that observed using PGE_2 as the agonist. This obviously contradicts the findings presented here. There are however several methodological differences which may account for this. In the experiments presented in this thesis, the agonist potencies were similar to that published by a variety of other researchers (Gryglewski *et al.*, 1987, Hecker *et al.*, 1990, Ney & Schrör, 1991) all of which were performed in the absence of phosphodiesterase inhibitors, and similar to those of Talpain *et al.* (1994) in the presence of the non-selective phosphodiesterase inhibitor IBMX. In the absence of IBMX, Talpain *et al.* (1994) found the agonists were less potent and few agonists reached a clear maximum response, partly because agonist concentrations of 10^{-5}M were not exceeded. Another difference is that the pre-incubation time for the agonists used in the present study were 5 min compared to 10 min (Talpain *et al.*, 1994) at 37°C prior to stimulation with fMLP. The time course of PGE_2-stimulated cAMP accumulation in this study was optimal at 5 min, either in the presence or absence of phosphodiesterase inhibition (see chapter 4), and in some experiments was slightly lower at 10 min compared to 5 min.

This suggests that in terms of adenylate cyclase activation, the pre-incubation period of 5 min was optimal for PGE_2 to ensure maximal elevation of cAMP levels on stimulation of O_2^- generation. In addition to this, the fMLP-stimulation period used in this study was 5 min, and not the 10 min adopted by Talpain *et al.* (1994). Nielson (1987) showed that fMLP-stimulated O_2^- generation by human neutrophils was biphasic; the first phase was terminated by 5-6 min and inhibited by isoprenaline, thereafter the second phase was initiated and the rate of O_2^- generation was increasing at 10 min and not inhibited by isoprenaline. In addition, the inhibitory effect of isoprenaline on fMLP-stimulated O_2^- generation was more pronounced after 5 than 15 min stimulation. Therefore only the first phase of fMLP-stimulated O_2^- generation was inhibited by β-agonists (increases in cAMP). Nielson demonstrated that PGE_2 had a similar effect, although it was less potent than isoprenaline as an inhibitor. These observations could account for the differences observed between the present study and that of Talpain *et al.*, in terms of potency and effectiveness of the agonists. Thus, in the latter, the assay conditions were not optimal for the study of these compounds.

AH 6809 enhanced fMLP-stimulated O_2^- generation in this present study, suggesting a stimulatory or priming effect on neutrophil function; but did not stimulate O_2^- generation itself. AH 6809 enhancement of fMLP-stimulated O_2^- generation by human neutrophils has subsequently been reported by Talpain *et al.* (1994). Stimulatory effects of AH 6809 in other tissues have been noted over a similar concentration range, such as spasmolytic activity on smooth muscle unrelated to its prostanoid receptor blocking activity observed by Coleman (1987). There have been many activities reported at these high concentrations of AH 6809 ($\geq 1 \times 10^{-5}$M); for instance antagonism of thromboxane synthase, antagonism of prostanoid TP receptors (Keery & Lumley, 1988) and inhibition of phosphodiesterase activity in mast cells (IC_{50} 2.6×10^{-5}M) although it is unclear which isoform is involved (Keery & Lumley, 1988). Inhibition of phosphodiesterase activity may explain AH 6809-mediated potentiation of

prostacyclin and NECA inhibition of human platelet aggregation (Keery & Lumley, 1988). This effect is the exact opposite to that observed by Talpain *et al.* (1994) in the human neutrophil. If an inhibition of phosphodiesterase does occur it may contribute to the lack of rightward shift of the PGE_2 O_2^- inhibition curve by 10^{-5}M AH 6809 observed in some donors.

More recently, AH 6809 (10^{-5}M) antagonism of PGE_2-induced relaxation of the rabbit ear artery, an EP_2-receptor mediated response (Humbles *et al.*, 1991), has been observed (personal communication from George Smith, Astra Charnwood, 1995). However antagonism was not observed in all preparations, and the degree of antagonism was small ($\leq$ 0.5 $\log_{10}$ units). Consequently, this effect of AH 6809 may not be attributable to antagonism of the EP_2 receptor, but to a non-prostanoid effect of AH 6809 as observed in other smooth muscle preparations (Coleman, 1987).

As EP_2 and EP_4 receptors mediate similar functions in many tissues, such as relaxation of smooth muscle; the effect of the EP_4 receptor antagonist AH23848B on PGE_2 was evaluated in the neutrophil. AH23848B is a more potent TP receptor antagonist (pA_2 7.8-8.3, Brittain *et al.*; 1984) than an EP_4 receptor antagonist (Coleman *et al.*, 1994a). Initially, due to its lack of potency, AH23848B was evaluated at 3×10^{-5}M, which would give approximately a $\log_{10}$ unit rightward shift of agonists at EP_4 receptors according to its literature pA_2 value (Coleman et *al.*, 1994a). However, under the assay conditions used, this concentration of AH23848B reduced the control fMLP O_2^- response to a level too small to quantify the effect of inhibitors accurately. Thereby, suggesting that AH23848B had a non-specific effect on neutrophil O_2^- generation or agonist activity at another inhibitory receptor on the human neutrophil. A lower concentration (10^{-5}M) which did not affect the control fMLP response did not antagonise the inhibitory effect of PGE_2 on fMLP-stimulated O_2^- generation or

PGE_2-stimulated cAMP accumulation. However, the lack of potency of this compound as an EP_4 receptor antagonist does not preclude the presence of EP_4 receptors on human neutrophils. Armstrong & Talpain (1994) reported however that AH23848B at $3x10^{-5}M$ did not significantly reduce PGE_2 stimulated cAMP generation in human neutrophils which supports the lack of EP_4 receptors on human neutrophils. Indeed, Talpain *et al.* (1994) reported that lower concentrations of AH23848B ($10^{-5}M$) increased fMLP-stimulated superoxide generation by human neutrophils and increased the potency of PGE_2, butaprost and NECA as inhibitors of fMLP-stimulated O_2^- generation. This effect on PGE_2 was not observed in the present study, which again may be accounted for by differences in protocol. Talpain *et al.* used a 10 min (37°C) pre-incubation period for the antagonists prior to addition of the agonists, whilst in this study the cells were pre-incubated for up to 60 min at room temperature to insure equilibrium had been reached. This is especially important in view of the low potency of some of the antagonists such as AH23848B, compared to the agonists. However the enhancement of the effects of other inhibitors of neutrophil activation observed by Talpain *et al.* (1994) may suggest AH23848B is a partial agonist, but this was rejected as AH23848B enhanced rather than inhibited fMLP-stimulated O_2^- generation. However in the present study, the converse was observed at $3x10^{-5}M$, which suggests that AH23848B may have some agonist activity although it is unclear which receptor is involved. This is not entirely unexpected as AH23848B has agonist activity at EP_4 receptor in the rabbit saphenous vein (Lydford *et al.*, manuscript in preparation).

In addition, this study has shown, as have others (Wheeldon & Vardey, 1993 and Armstrong & Talpain, 1994), that the EP_2 selective agonist AH13205 was just 30 fold less potent than PGE_2 on the human neutrophil. This potency difference is consistent with the presence of EP_2 receptors and the absence of EP_4 receptors, as AH13205 is 11,000 less potent than PGE_2 in EP_4 receptor containing tissues (Coleman *et al.*, 1994a). However, as there are no

reported selective agonists or more potent selective antagonists, for this most recently described subtype, the EP_4 receptor; the presence of EP_4 receptors on human neutrophils cannot be conclusively demonstrated.

This study has shown that contrary to the established classification, AH 6809 is an antagonist at the human EP_2 receptor and not a selective EP_1 or DP receptor antagonist. The data from the human neutrophil provides evidence that this EP receptor may be different to other species or perhaps represents a novel subtype. However, the lack of detailed information in the literature on the actions of AH 6809 in EP_2 receptor containing tissues especially human, means that experimental differences cannot be excluded. This is especially important in the context that EP_2 and EP_4 receptors can mediate similar responses in the same tissue; and at the time when AH 6809 was described as an EP_1 selective antagonist (Coleman *et al.*, 1987), EP_4 receptors were unknown. So it is possible that the lack of antagonism observed at EP_2 receptor containing tissues could be due to the presence of EP_4 receptors masking any antagonism. Differences in experimental protocol could also have masked AH 6809 antagonism of EP_2 receptors, for instance if AH 6809 was added to the tissue to block any effects of DP and EP_1 receptors in the tissue prior to testing any EP agonists, the EP_2 receptors could also have been blocked. In addition, in some preparations AH 6809 may have activity which precludes clear interpretation of its prostanoid antagonist activity, such as platelets and smooth muscle preparations. The use of AH 6809 is also complicated by its lipophilicity. AH 6809 is extremely highly plasma protein bound, approximately 97% is bound in 4% BSA (Coleman *et al.*, 1990) and therefore non-specific binding to protein and diffusion may pose a variable problem between tissue preparations. The lipophilicity of AH 6809 would present less of a problem for cell preparations, unless there are protein supplements or a large number of non-participating cells in the assay.

It can be said then, that the characterization of prostanoid receptors in general is fraught with

difficulties. Not least because most tissues and cells possess at least two different prostanoid receptors, for example, the human neutrophil has EP_2 and DP receptors (and possibly EP_3), the human platelet has DP, IP and TP receptors, the guinea pig trachea has EP_1 and EP_2 receptors and the rabbit ear artery has EP_2 and EP_3 receptors.

3.3.2 Biological activity of PGE metabolites

Natural prostaglandins are rapidly metabolised and labile, the plasma half-life of a prostaglandin on reaching the blood stream is estimated as less than 1 min. The initially formed metabolites are the 15-keto-13,14-dihydro compounds which appear within 1 min and have a half-life of about 10 min in the blood. For the PGE compounds this is the predominant metabolic pathway, and other metabolites are subsequently formed via β-oxidation or ω-oxidation, but there is also interconversion between metabolites of the PGE and PGF compounds. The PGA and PGB compounds are products of non-enzymic degradation formed by dehydration of the PGE molecules, by the action of acid and base respectively. Their presence in biological samples is generally considered to be artifacts formed *ex-vivo* during storage prior to measurement (Horton, 1979). Nevertheless, an enzyme has been identified which can catalyse the isomerisation of PGA_2 to PGC_2 (Jones & Cammock, 1973) followed by isomerisation to PGB_2. Thus the indications are that these molecules may be formed *in vivo*; and as the dehydration of PGE moieties into PGA and PGB compounds is especially rapid in the presence of albumin, their formation in inflamed tissue, such as sites of plasma leakage and oedema, is a distinct possibility. The biological activity of PGE_0, PGA_1, PGA_2, PGB_1 and PGB_2 and other products of PGE metabolism and degradation are physiologically and pharmacologically significant. Some of these metabolites are also more stable than their parent molecules; these together suggest that the duration of the biological activity of PGE_1 and PGE_2 may extend beyond the lifetime of the parent. Also of significance, is the activity of these metabolites at other prostanoid receptors

on leukocytes and in other tissues and how this reflects on the physiological and pathological effects of prostaglandins in normal and disease conditions.

3.3.3 Inhibitory prostanoid DP receptors on human neutrophils

Prostanoid DP receptor agonists also inhibit human neutrophil activation, and one of the most potent prostanoid agonists evaluated in this study is a DP agonist, BW245C. However, the human neutrophil prostanoid DP receptor does not appear to be pharmacologically different to the DP receptor found in other tissues (Wheeldon & Vardey, 1993 and the present study) in terms of its sensitivity to prostanoid DP agonists or antagonists. There are preliminary reports of evidence which suggests subtyping of prostanoid DP receptors, for instance BW A868C (selective DP receptor antagonist) appearing to interact with 2 receptors in the rabbit saphenous vein (Lydford *et al.*, 1994).

In summary, the pharmacological characterization of the inhibitory prostanoid EP_2 receptor on the human neutrophil using fMLP-stimulated O_2^- generation as the assay system has been described in this chapter. Furthermore, the same agonist potency order exists for cAMP elevation, and PGE_2 stimulated increases in cAMP are also inhibited by AH 6809.

CHAPTER 4

EFFECT OF PGE_2 ON cAMP LEVELS IN HUMAN NEUTROPHILS AND cAMP MODULATION OF $[Ca^{2+}]_i$ AND SUPEROXIDE GENERATION

4.1 INTRODUCTION

4.1.1 Modulation of human neutrophil activation by cAMP

Many inhibitors of leukocyte activation, such as phosphodiesterase inhibitors (PDEIs), prostaglandins D_2 and E_2 (PGD_2 and PGE_2) and β-adrenoceptor agonists, elicit an increase in cAMP in the target cell. All of these agents inhibit human neutrophil activation and simultaneously elevate cAMP levels. The latter has been proposed as a common mechanism of action to explain their inhibitory activities.

Amongst the most intensively studied inhibitors are PGE_2 (Gryglewski *et al.*, 1987, Rossi & O'Flaherty, 1989, Hecker *et al.*, 1990) and PDEIs (Nielson *et al.*, 1990, Ho *et al.*, 1990, Schudt *et al.*, 1991). Both types of agents elevate cAMP levels; PGE_2 by prostanoid EP receptor mediated activation of adenylate cyclase catalyzing the formation of cAMP from ATP, and PDEI's by inhibiting cAMP metabolism. The phosphodiesterase activity in human neutrophils is predominately type IV, the cGMP-insensitive cAMP-selective isozyme (Nielson *et al.*, 1990, Wright *et al.*, 1990). PGE_2 and PDEIs inhibit fMLP-stimulated superoxide (O_2^-) generation and increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) by human neutrophils. At present, it is unclear if cAMP-elevating agents inhibit human neutrophil activation by attenuating stimuli-induced increases in $[Ca^{2+}]_i$, and if they do, which mechanisms are involved.

Interestingly, the neutrophil activator, formyl-methionyl-leucyl-phenylalanine (fMLP), has been reported to induce a transient (15-30 second) increase in cAMP levels (doubling of basal levels). The transience of this fMLP-stimulated cAMP increase is Ca^{2+}-dependent and is abolished in the presence of PDEIs (Verghese *et al.*, 1985). However, the precise mechanism is unknown, but may represent a physiological process implemented by neutrophils to terminate stimulus transduction signals (Verghese *et al.*, 1985). In which case, cAMP elevation may be a global cellular mechanism utilised by both inhibitors (PDEIs and PGE_2) and stimulators to attenuate neutrophil activation. The inhibitors may exert their suppressive effects by preempting and/or potentiating the

stimulus (fMLP)-induced cAMP elevation associated with inactivation of cellular responses (Verghese *et al.*, 1985).

4.1.2 Role of calcium in human neutrophil activation

Neutrophil activation by receptor-mediated stimuli is invariably associated with an increase in cytosolic free Ca^{2+} ion concentration ($[Ca^{2+}]_i$). An increase in $[Ca^{2+}]_i$ is essential but not sufficient for activation of neutrophils. fMLP-stimulated degranulation and O_2^- generation is abolished by depleting stored Ca^{2+} ions and buffering $[Ca^{2+}]_i$ with intracellular Ca^{2+} chelators (O'Flaherty *et al.*, 1991). Indeed, many neutrophil functions can be stimulated by agents which do not increase $[Ca^{2+}]_i$ including phorbol esters (PMA), suggesting that protein kinase C (PKC) activation is also involved in eliciting neutrophil responses (Lew *et al.*, 1984, Nielson, 1987).

fMLP, the bacterial peptide, is one of the best known and widely used stimuli in human neutrophil function studies. fMLP and the complement fragment C5a are described as complete stimuli/secretagogues. They are able to elicit the full range of neutrophil responses - aggregation, chemotaxis, exocytosis, O_2^- generation and shape change. Incomplete stimuli/secretagogues such as LTB_4 and interleukin 8 (IL-8) are potent chemotactic agents, but are extremely poor stimulators of superoxide generation or degranulation (Wymann *et al.*, 1987, Baggiolini *et al.*, 1989).

fMLP activates a specific transmembrane receptor coupled to phospholipase C and subsequently increases intracellular free calcium levels $[Ca^{2+}]_i$ and activates protein kinase C (PKC). The characteristics of the fMLP-stimulated biphasic increase in $[Ca^{2+}]_i$ are well documented (Sage *et al.*, 1990, Hecker *et al.*, 1990). Following the addition of fMLP to neutrophils; there is an immediate rapid transient increase peaking between 10-20s which declines into a plateau phase which remains elevated above basal which returns only slowly to the unstimulated $[Ca^{2+}]_i$ (by approximately 10 min). The first phase has been attributed to the mobilisation of Ca^{2+} ions from intracellular endoplasmic stores

(Lew *et al.*, 1984, Sage *et al.*, 1990) as it is unchanged in the absence of extracellular Ca^{2+} ions. The protracted phase is due to the influx of Ca^{2+} ions via cation channels in the plasma membrane and abolished by the removal of extracellular Ca^{2+} ions (Sage *et al.*, 1990).

Initial experiments, measuring changes in $[Ca^{2+}]_i$ appeared to show that the onset of fMLP-stimulated NADPH-oxidase activation (2.4 seconds) preceded the onset of the increase in $[Ca^{2+}]_i$ by 2 seconds (Wymann *et al.*, 1987). This study, as with most studies measuring neutrophil $[Ca^{2+}]_i$, used conventional fluorimetry with cell suspensions in cuvettes. This technique lacks the resolution of the initial changes in fluorescence mainly due to the a lag phase in detection of changes in fluorescence required for mixing of any additions, such as stimuli to the cell suspensions. However, the use of stopped-flow fluorimetry allowed the early subsecond kinetics of fMLP-stimulated increases in $[Ca^{2+}]_i$ to be determined (Sage *et al.*, 1990). With the stopped-flow technique, the onset of fMLP-stimulated increase in $[Ca^{2+}]_i$ was found to be 1-1.3 seconds after fMLP addition. Thus, in the human neutrophil, fMLP-stimulated increases in $[Ca^{2+}]_i$ precede that of NADPH-oxidase activation; suggesting that increases in $[Ca^{2+}]_i$ may be involved in fMLP-stimulated superoxide generation. Not only does the increase in $[Ca^{2+}]_i$ precede the respiratory burst; but it also precedes neutrophil degranulation and aggregation (Korchak *et al.*, 1984a and b). This study showed that fMLP-stimulated ^{45}Ca uptake (an index of Ca permeability) in neutrophils occurred prior to superoxide generation, degranulation and aggregation.

It would appear then that an increase in $[Ca^{2+}]_i$ is involved in many if not all neutrophil functions, and the modulation of inflammatory stimuli (e.g. fMLP)-induced increases in $[Ca^{2+}]_i$ would be anti-inflammatory.

4.1.3 Aims

The aims of this chapter were:-

1. To determine the relationship between PGE_2-stimulated cAMP elevation and inhibition of human neutrophil activation; by studying how PGE_2 and PDEIs alone, and in combination, modulate neutrophil cAMP levels and fMLP-stimulated superoxide generation.

2. To determine how cAMP elevation modulates increases in cytosolic free calcium, an event associated with neutrophil activation; by studying the effect of PGE_2 and the selective type IV PDEI, rolipram on fMLP-stimulated increases in $[Ca^{2+}]_i$ in human neutrophils.

3. Establish whether cAMP-elevating agents can modulate Ca^{2+} and Mn^{2+} (a Ca^{2+} surrogate) entry in human neutrophils; and whether this could contribute to their inhibitory effect on fMLP-stimulated superoxide generation.

4.2 RESULTS

4.2.1 Effect of PDEIs and PGE_2 on cAMP levels in human neutrophils

Rolipram concentration-dependently stimulated small increases in neutrophil cAMP levels measured after 5 min stimulation (equal to the pre-incubation period in the superoxide assay) (Fig 4.1) and were higher at 10 min. IBMX did not stimulate a significant increase in cAMP above basal levels measured after 10 min. Rolipram and IBMX, at concentrations which inhibit fMLP-stimulated O_2^- generation, stimulated small or no increase in neutrophil cAMP levels. Significant increases in cAMP were only observed at supramaximal inhibitory concentrations of PDEI (Schudt *et al.*, 1991). In fMLP (10^{-7}M)-stimulated neutrophils, rolipram, the selective type IV PDEI increased cAMP levels above those in unstimulated cells (Table 4.1). In contrast, the non-selective PDEI, IBMX did not increase cAMP levels in either unstimulated or fMLP-stimulated neutrophils (Table 4.1).

PGE_2 stimulated increases in cAMP levels were measured in the presence of IBMX ($5x10^{-4}$M) after 2, 5 or 10 min stimulation (n=3) and were not significantly different (Fig 4.2). In contrast, forskolin was a poor stimulus of cAMP accumulation in human neutrophils in the presence of IBMX. Even after 10 min stimulation with forskolin (10^{-4}M), the cAMP levels were only 2.13±0.73 pmol cAMP 10^6 neutrophils^{-1} compared with basal levels of 0.83±0.23 pmol cAMP 10^6 neutrophils^{-1} (n=6). In fMLP (10^{-7}M)-stimulated neutrophils, PGE_2 stimulated higher levels of cAMP accumulation (measured over the same time course as the superoxide assay) (Table 4.2) but the $p[A_{50}]$ for PGE_2 was similar in the absence or presence of fMLP (Fig 4.3). The cAMP-potentiating effects of fMLP on PGE_2-stimulated cAMP accumulation were not as dramatic as those observed on rolipram stimulated cAMP increases.

In contrast, cytochalasin B (5μg ml^{-1}) had no effect on cAMP levels in either unstimulated or fMLP-stimulated neutrophils (Table 4.2) and did not alter the levels of cAMP accumulated on stimulation of human neutrophils by PGE_2 (Table 4.2).

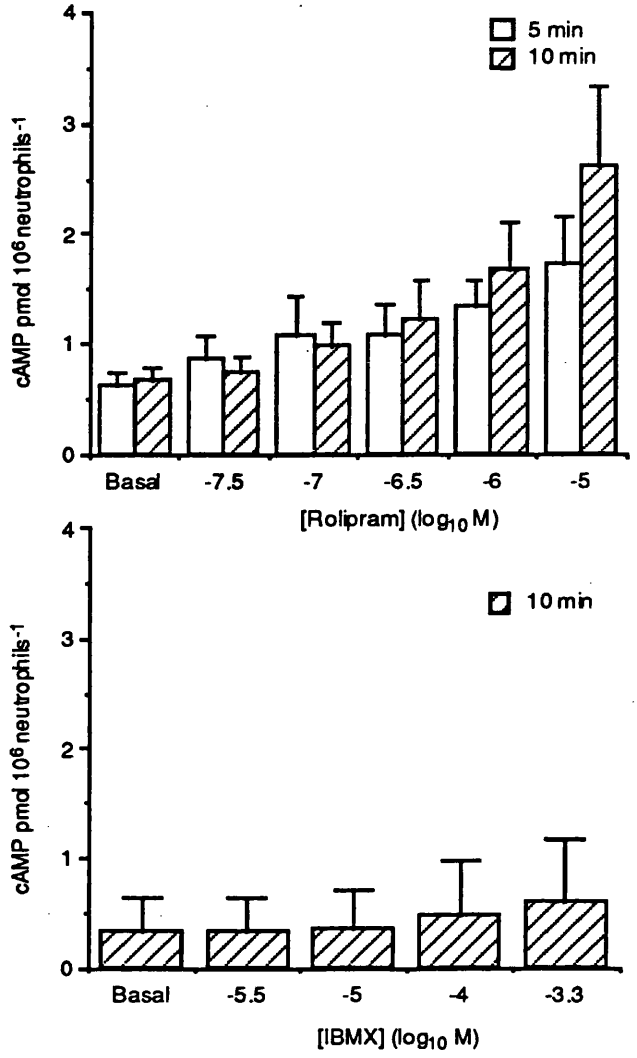


Fig 4.1 Effect of rolipram on human neutrophil cAMP levels measured at 5 min and 10 min (upper panel) and IBMX measured at 10 min (lower panel). Prewarmed neutrophils (10^6 per determination, 5 min, 37°C) were incubated with the PDEIs for 5 min or 10 min. Results shown are the mean±s.e.m cAMP levels (pmol cAMP 10^6 neutrophils^{-1}) from 3 separate experiments performed in duplicate.

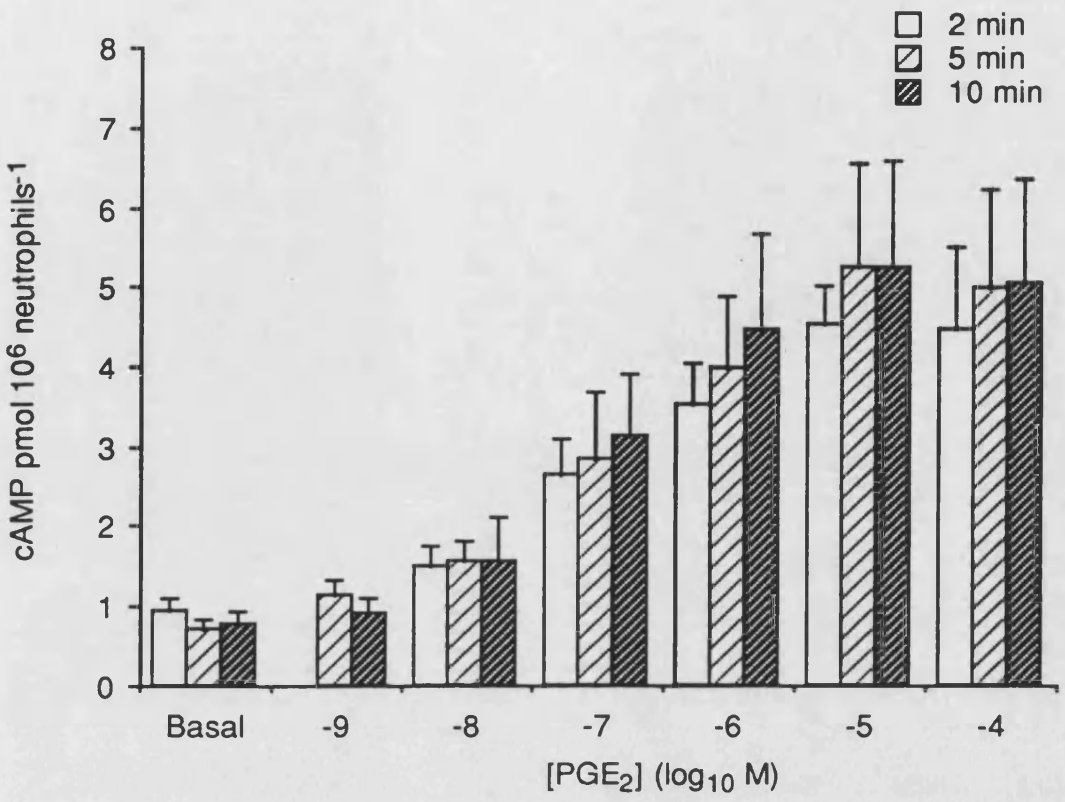


Fig 4.2 Time course of PGE_2-stimulated cAMP accumulation by human neutrophils. Neutrophils (10^6 per determination) were preincubated with IBMX ($5x10^{-4}$M, 5 min, 37°C) prior to stimulation with PGE_2 for 2, 5 or 10 min (37°C). Results shown are mean±s.e.m increases in cAMP (pmol 10^6 neutrophils^{-1}) of 3 separate experiments performed in duplicate.

[Rolipram] (log_{10} M)	+ Cyt B cAMP pmol 10^6 neutrophils^{-1}	+ Cyt B/fMLP cAMP pmol 10^6 neutrophils^{-1}
Basal	0.52±0.16	0.49±0.17
-8.5	0.46±0.15	0.60±0.27
-8	0.56±0.19	0.61±0.23
-7.5	0.78±0.26	0.78±0.33
-7	0.85±0.37	1.44±0.82
-6.5	0.99±0.41	2.17±1.43
-6	1.01±0.47	3.07±1.98
-5	1.39±0.61	4.89±2.87

[IBMX] (log_{10} M)	+ Cyt B/fMLP cAMP pmol 10^6 neutrophils^{-1}
Basal	0.42±0.13
-5.5	0.49±0.12
-5	0.57±0.16
-4	0.97±0.28

Table 4.1 Effect of rolipram and IBMX on cAMP levels of human neutrophils stimulated with fMLP (10^{-7}M). Neutrophils (10^6 per determination) were incubated with rolipram or IBMX in the presence of cytochalasin B (5 min, 37°C) prior to stimulation with buffer or fMLP (5 min, 37°C). Results shown are the mean±s.e.m neutrophil cAMP levels (pmol cAMP 10^6 neutrophils^{-1}) of 3 separate experiments performed in duplicate.

	Control	Cyt B	fMLP	Cyt B/fMLP
Basal cAMP pmol 10^6 neutrophils^{-1}	1.02±0.2	1.14±0.09	2.06±0.76	2.35±0.62
PGE_2 max cAMP pmol 10^6 neutrophils^{-1}	16.76±2.84	16.47±2.08	42.62±13.77	37.06±10.55
PGE_2 p[A_{50}]	6.83±0.19	6.53±0.08	6.58±0.40	6.93±0.16

Table 4.2 Summary of effects of cytochalasin B (5μg ml^{-1}) and fMLP (10^{-7}M) on PGE_2 cAMP elevation in human neutrophils. Neutrophils (10^6 per determination) were preincubated with IBMX ($5x10^{-4}$M) and PGE_2 (5 min, 37°C) in the absence or presence of cytochalasin B prior to the addition of buffer or fMLP (5 min, 37°C). Results shown are mean±s.e.m of data from 3 separate experiments performed in duplicate.

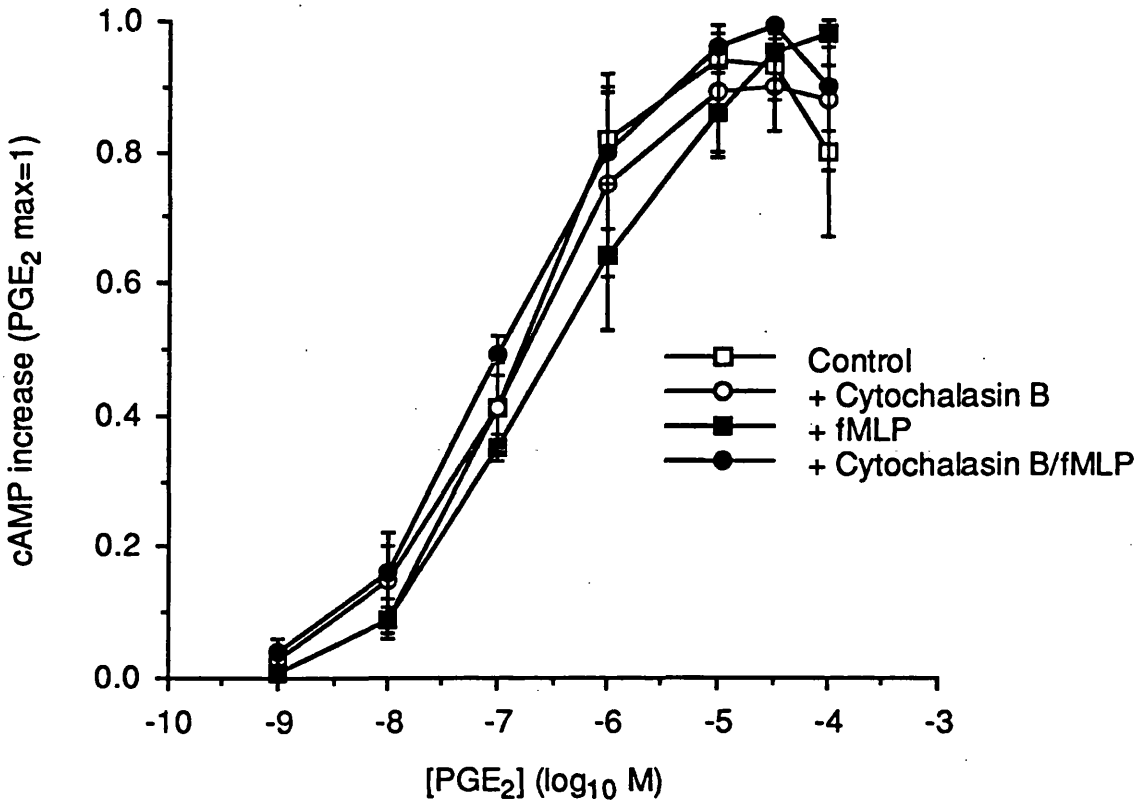


Fig 4.3 PGE_2-stimulated cAMP accumulation by human neutrophils - effect of cytochalasin B (5μg ml^{-1}) and fMLP (10^{-7}M)-stimulation. Neutrophils (10^6 per determination) were preincubated with IBMX ($5x10^{-4}$M, 5 min, 37°C) prior to addition of PGE_2 (5 min, 37°C) in the absence or presence of cytochalasin B (5μg ml^{-1}) and stimulated with fMLP or buffer (5 min, 37°C). Results shown are mean±s.e.m cAMP increase normalised with respect to the PGE_2 maximum (=1) for each treatment measured after the fMLP stimulation period, of 3 separate experiments performed in duplicate.

4.2.2 Effect of PDEIs and PGE_2 on fMLP-stimulated superoxide generation by human neutrophils

The phosphodiesterase inhibitors (PDEIs), IBMX (non-selective), rolipram and RO 20-1724 (both selective for the type IV isoenzyme) inhibited fMLP (10^{-7}M)-stimulated O_2^- generation with $p[A_{50}]$ values of 5.0±0.2 (n=8), 7.4±0.1 (n=4) and 6.9±0.1 (n=6) respectively. Clear maximum responses were reached by all of these PDEIs, 75.1±2.6% by IBMX ($5x10^{-4}$M), 72.7±4.3% by rolipram (10^{-5}M) and 72.3±2.6% by RO 20-1724 ($3x10^{-5}$M). Milrinone, however, a type III selective PDEI (Harrison *et al.*, 1986, Lindgren *et al.*, 1990), was a poor inhibitor of fMLP-stimulated O_2^- generation. A clear maximum response was not reached even at 10^{-4}M (50.0±2.1%, n=3), so a pIC_{50} of 4.2±0.1 was calculated as a $p[A_{50}]$ could not be estimated. The concentration-effect curves for IBMX, rolipram, RO 20-1724 and milrinone inhibition of fMLP-stimulated O_2^- generation by human neutrophils are shown in Fig 4.4.

The effect of combining PGE_2 and PDEIs on fMLP-stimulated O_2^- generation by human neutrophils was studied in order to provide evidence for cAMP elevation as the transducer pathway via which PGE_2 mediates inhibition of neutrophil activation. The PDEIs IBMX (non-selective) and RO 20-1724 (type IV selective), at $3x10^{-6}$-$1x10^{-4}$M and $3x10^{-8}$-$1x10^{-5}$M respectively, concentrations which inhibit fMLP-stimulated O_2^- generation, potentiated PGE_2-mediated inhibition of fMLP-stimulated O_2^- generation. The $p[A_{50}]$ of PGE_2 was left-shifted concentration-dependently by IBMX and RO 20-1724 (Fig 4.5 and Table 4.3). PGA_1 (another prostanoid EP receptor agonist)-inhibition of fMLP-stimulated O_2^- generation was similarly potentiated by IBMX (Table 4.4).

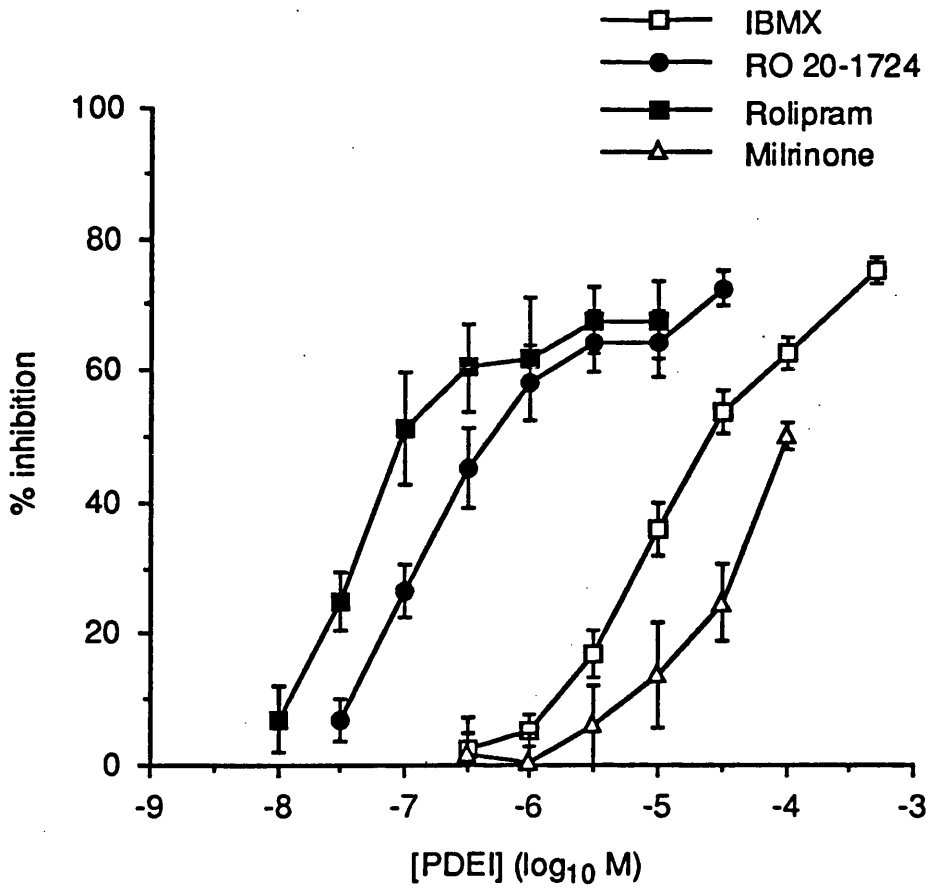


Fig 4.4 Effect of PDEIs; IBMX, rolipram, RO 20-1724 and milrinone, on fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with PDEIs in the presence of cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP for 5 min (37°C). Results shown are mean±s.e.m % inhibition of control fMLP response of 8 (IBMX), 4 (rolipram), 6 (RO 20-1724) and 3 (milrinone) separate experiments performed in duplicate.

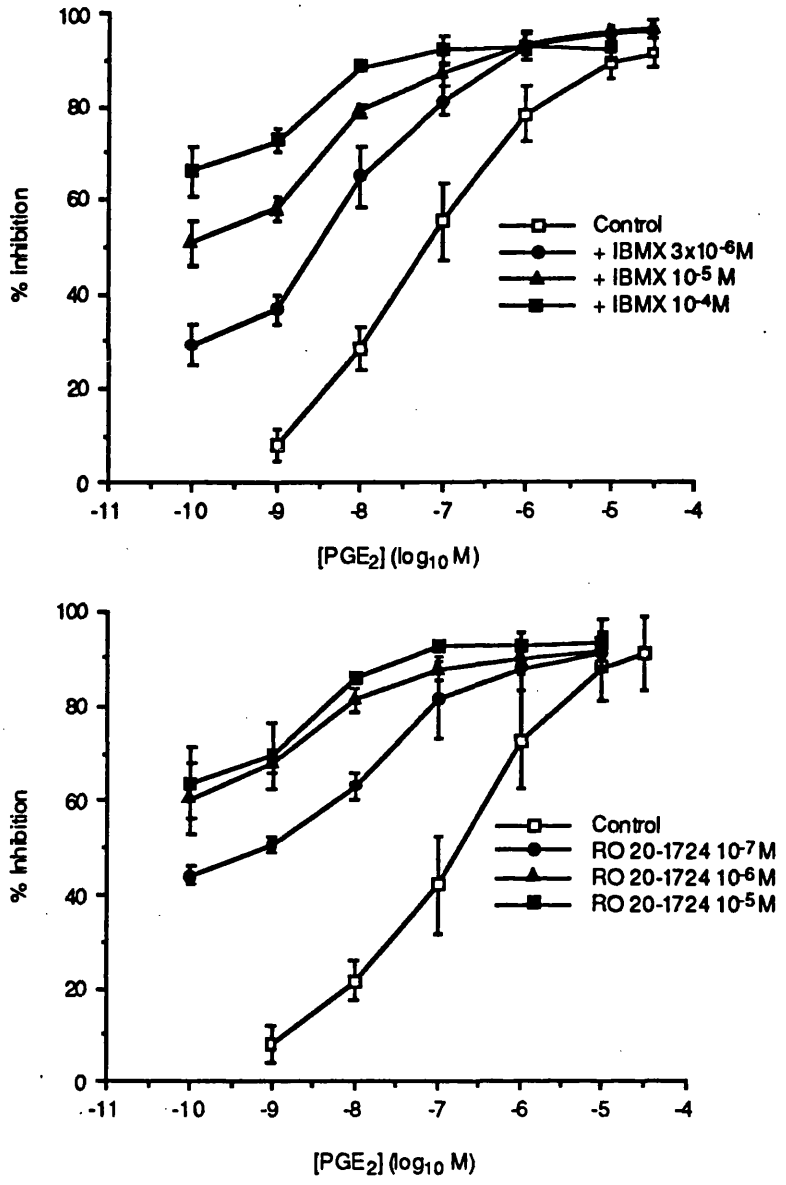


Fig 4.5 Effect of IBMX (upper panel) and RO 20-1724 (lower panel) on PGE_2-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were simultaneously incubated with cytochalasin B (5μg ml^{-1}), PGE_2 +/-IBMX or RO 20-1724 (5 min, 37°C) prior to fMLP stimulation (5 min, 37°C). Results shown are mean±s.e.m % inhibition of the control fMLP response of 5 (IBMX) and 3 (RO 20-1724) separate experiments performed in duplicate. Inhibition by IBMX alone was 29.7±3.7% ($3x10^{-6}$M), 50.6±4.3% (10^{-5}M) and 64.0±7.2% (10^{-4}M), and RO 20-1724 alone were 45.5±7.5% (10^{-7}M), 62.3±6.7% (10^{-6}M) and 65.7±10.1% (10^{-5}M) respectively.

PGE_2 Treatment	PGE_2 p[A_{50}]	Maximum % Inhibition
Control	7.37±0.16	91.4±3.3
+IBMX $1x10^{-6}M$	7.65±0.35	96.5±0.5
+IBMX $3x10^{-6}M$	8.12±0.02**	96.3±1.1
+IBMX $1x10^{-5}M$	8.24±0.12**	97.2±1.2
+IBMX $1x10^{-4}M$	8.41±0.11**	93.3±3.9
Control	7.07±0.11	91.0±4.0
+RO 20-1724 $1x10^{-7}M$	7.76±0.24	91.0±4.0
+RO 20-1724 $1x10^{-6}M$	8.37±0.33*	91.7±3.5
+RO 20-1724 $1x10^{-5}M$	8.38±0.16*	95.7±3.2

Table 4.3 Effect of IBMX and RO 20-1724 on PGE_2 inhibition of fMLP-stimulated O_2^- generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with PGE_2+/-IBMX or RO 20-1724 in the presence of cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP ($10^{-7}M$) for 5 min (37°C). Results shown are mean±sem % inhibition of the control fMLP response from 5 separate experiments for the effects of IBMX (shown in the upper part of the table) and 3 separate experiments for RO 20-1724 (in the lower part of the table), each performed in duplicate. Statistically significant changes compared with control data indicated by * for $P<0.05$ and ** for $P<0.01$ (paired Student's t-test).

Treatment	PGA_1 $p[A_{50}]$	Maximum % Inhibition
Control	6.93±0.15	89.0±2.4
+IBMX $3x10^{-6}M$	7.20±0.20	93.5±1.5
+IBMX $1x10^{-5}M$	7.37±0.23*	94.3±2.9

Table 4.4 Effect of IBMX on PGA_1 inhibition of fMLP ($10^{-7}M$)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with PGA_1+/-IBMX in the presence of cytochalasin B (5µg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP ($10^{-7}M$) for 5 min (37°C). Results shown are mean±sem % inhibition of the control fMLP response from 3 separate experiments performed in duplicate.Statistically significant changes compared with control data indicated by * for $P<0.05$ (paired Student's t-test).

4.2.3 Effect of adenylate cyclase and protein kinase A inhibitors on superoxide generation by human neutrophils

SQ 22356, the adenylate cyclase inhibitor (10^{-4}M, Tamaoki *et al.*, 1993), when pre-incubated with the neutrophils for 20 min prior to addition of PGE_2 or IBMX reduced the control fMLP O_2^- response by 15% and 22% in 2 donors. Unexpectedly, SQ 22356 did however left-shift the PGE_2 inhibition curve $p[A_{50}]$ values in both experiments from 7.5 to 8.1 and 7.0 to 8.2 with no effect on the PGE_2 maximum (91% and 87% in the absence, 99% and 98% in the presence of SQ 22356). The IBMX inhibition curve was unaffected in one experiment ($p[A_{50}]$ 5.1 and 5.0 in the absence and presence of SQ 22356 respectively) and was right-shifted in the second experiment from 5.3 to 4.7 by SQ 22356.

H89, the protein kinase A inhibitor, had no effect on fMLP (10^{-7}M)-stimulated O_2^- generation at 3×10^{-6}M, 10^{-5}M and 3×10^{-5}M. These concentrations have a selective effect on protein kinase A dependent processes without affecting protein kinase G responses in intact cells (Chijiwa *et al.*, 1990). The fMLP responses in the presence of H89 were 109±2%, 108±5% and 99±6% of the control fMLP response respectively (n=3). There was, however, a concentration-dependent reversal of PGE_2 (10^{-6}M) and IBMX (3×10^{-5}M) inhibition of fMLP-stimulated superoxide generation (Fig 4.6).

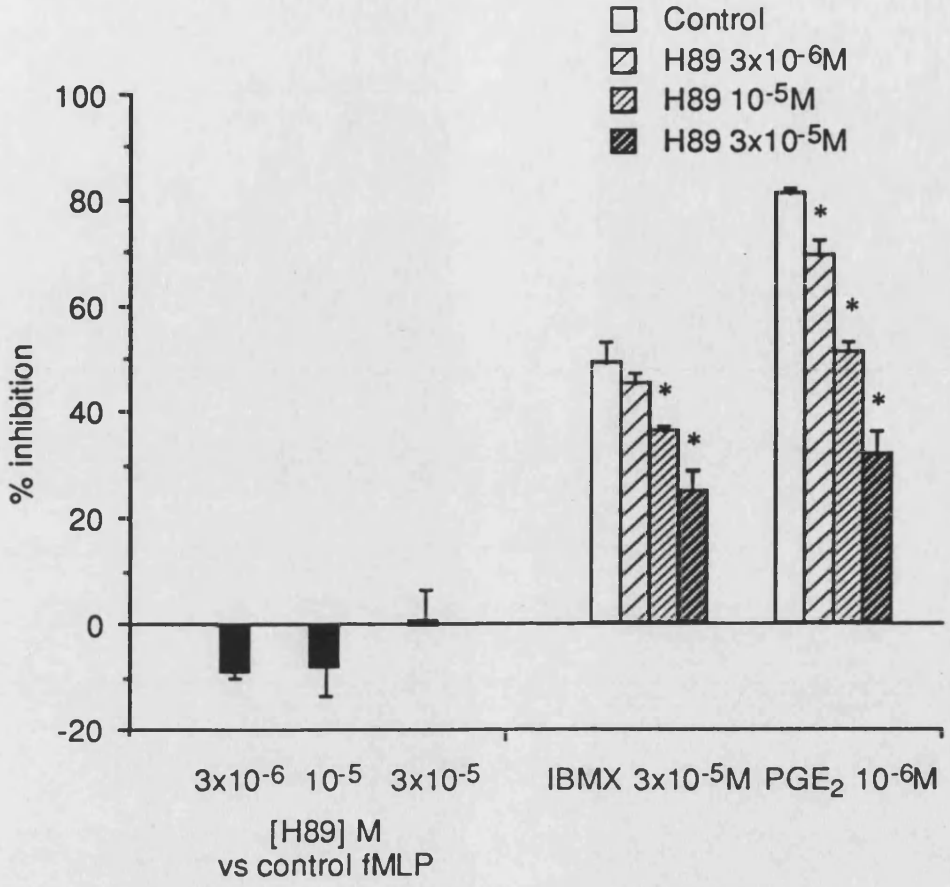


Fig 4.6 Effect of protein kinase A inhibitor H89 on PGE_2 (10^{-6}M) and IBMX ($3x10^{-5}$M)-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were pretreated with H89 (20 min, room temperature) prior to the addition of PGE_2 or IBMX (5 min, 37°C) and then stimulated with fMLP (5 min, 37°C). Effect of H89 on control fMLP-stimulated superoxide generation are shown by the filled bars on the left of the histogram. Results shown are mean±s.e.m % inhibition of 3 separate experiments performed in duplicate. Statistical significance compared with control indicated by an asterisk representing $P<0.05$ as determined by Student's t-test.

4.2.4 Effect of divalent cation chelators and Ca^{2+} entry blockers on fMLP-stimulated superoxide generation by human neutrophils

4.2.4.1 Effect of EDTA and EGTA

The presence of extracellular divalent cation chelators EDTA (Ca^{2+} and Mg^{2+} ions) or EGTA (Ca^{2+} ions only) attenuated O_2^- generation induced by fMLP (Fig 4.7). fMLP (10^{-7}M)-stimulated O_2^- generation was reduced in the presence of EDTA and EGTA by 48.6±5.1% and 62.1±4.6% respectively (n=3, $p<0.05$ for both), suggesting that fMLP-stimulated O_2^- generation is partially dependent on Ca^{2+} influx. However the fMLP $p[A_{50}]$ 7.8±0.1 was not significantly different in the presence of EDTA (1mM) or EGTA (0.95mM), 7.8±0.1 and 7.6±0.1 respectively (n=3, $p>0.05$).

In addition to this, PGE_2 and the PDEIs (rolipram and RO 20-1724) inhibited fMLP-stimulated O_2^- generation in the presence of EGTA (Fig 4.8). Indeed, PGE_2 was more potent against fMLP-stimulated O_2^- generation in the presence of EGTA. The PGE_2 $p[A_{50}]$ of 7.3±0.2 in the absence of EGTA was left-shifted in the presence of EGTA to 8.1±0.2 (n=3, $p<0.05$), whilst the potency of the PDEIs were not significantly different in the absence or presence of EGTA. The $p[A_{50}]$ of rolipram was 7.8±0.3 and 7.7±0.2, and RO 20-1724 was 7.1±0.1 and 6.9±0.2, in the absence and presence of EGTA respectively (n=3).

4.2.4.2 Effect of Ca^{2+} entry blockers; econazole and Ni^{2+} ions

The cytochrome P450 inhibitor, econazole, at 3×10^{-6}M and 10^{-5}M potentiated O_2^- generation stimulated by lower concentrations of fMLP (2×10^{-8}M) by 37±13% and 59±4% respectively, but did not inhibit the response to 10^{-7}M fMLP (105±3% and 90±2% of the control response) (mean±range of 2 separate experiments). Higher concentrations of econazole (3×10^{-5}M and 10^{-4}M), blocked O_2^- generation stimulated by fMLP at both 2×10^{-8}M (79±17% and 81±12% respectively) and 10^{-7}M (95±3% and 95±2% respectively) (mean±range of 2 separate experiments).

Ni^{2+} ions block receptor-mediated Ca^{2+} entry (RMCE) and voltage operated Ca^{2+} channels (VOC). The addition of extracellular $NiCl_2$ (3mM) as a source of Ni^{2+} ions, did not inhibit fMLP (10^{-7}M) stimulated O_2^- generation. There was however a degree of inhibition of the O_2^- response stimulated by lower concentrations of fMLP (10^{-8}M) (Fig 4.9).

4.2.4.3 The effect of inhibition of Ca^{2+} entry on thapsigargin-stimulated superoxide generation by human neutrophils

Thapsigargin, the endoplasmic reticulum Ca^{2+}-ATPase inhibitor (10^{-7}M-10^{-6}M), stimulated O_2^- generation by human neutrophils which was completely suppressed by the presence of 1mM EGTA (Fig 4.10). Thapsigargin ($1x10^{-6}$M)-stimulated O_2^- generation (14.8±5.3 nmol O_2^- 10^6 neutrophils^{-1}) was partially inhibited by PGE_2 (10^{-5}M) and rolipram (10^{-6}M) (Fig 4.11). Unlike fMLP, O_2^- generation stimulated by thapsigargin (10^{-7}M-10^{-6}M) was completely abolished by the presence of $NiCl_2$ (3mM) (Fig 4.12).

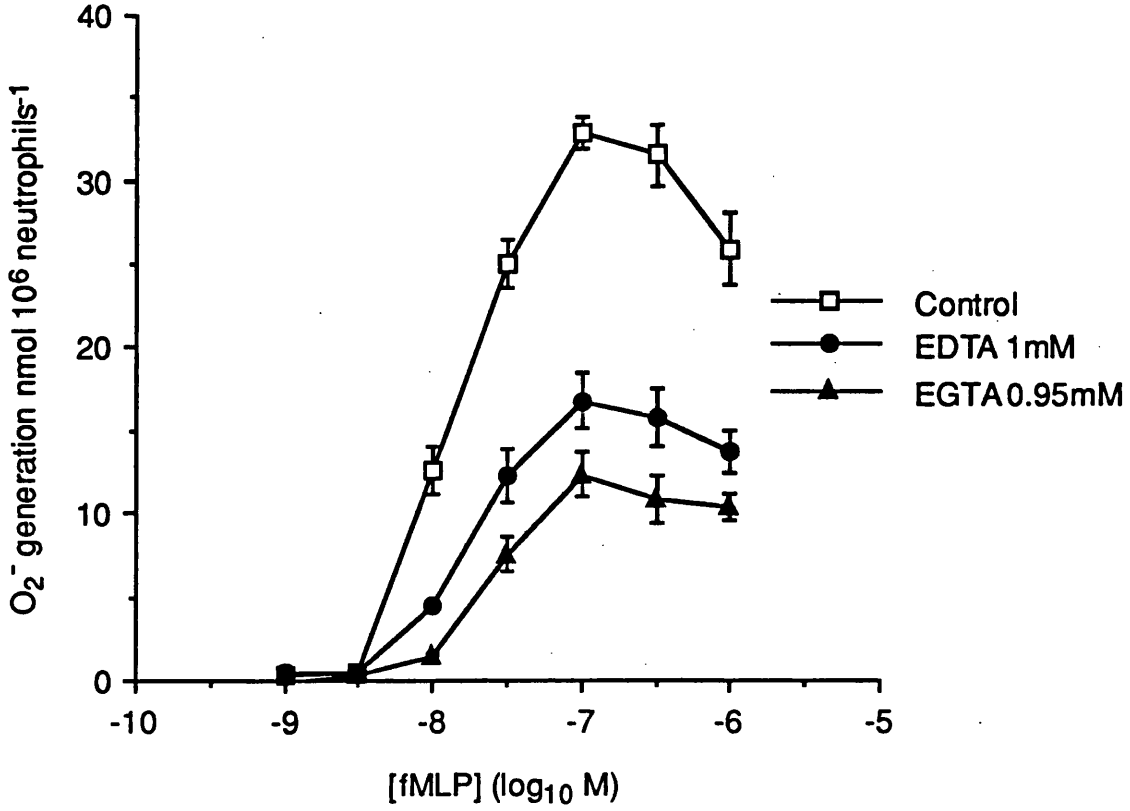
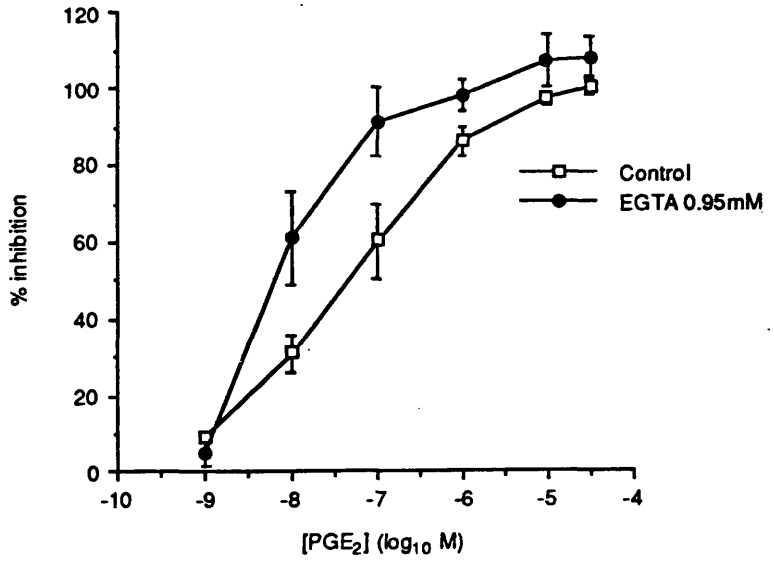
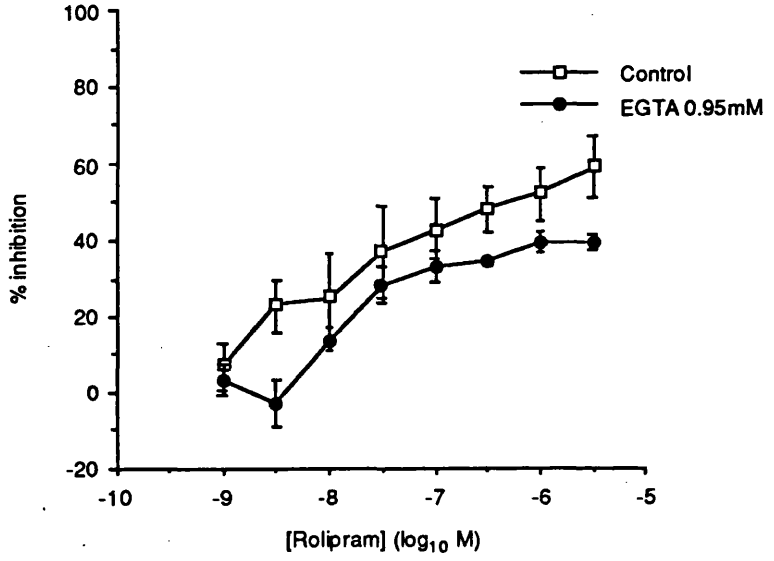
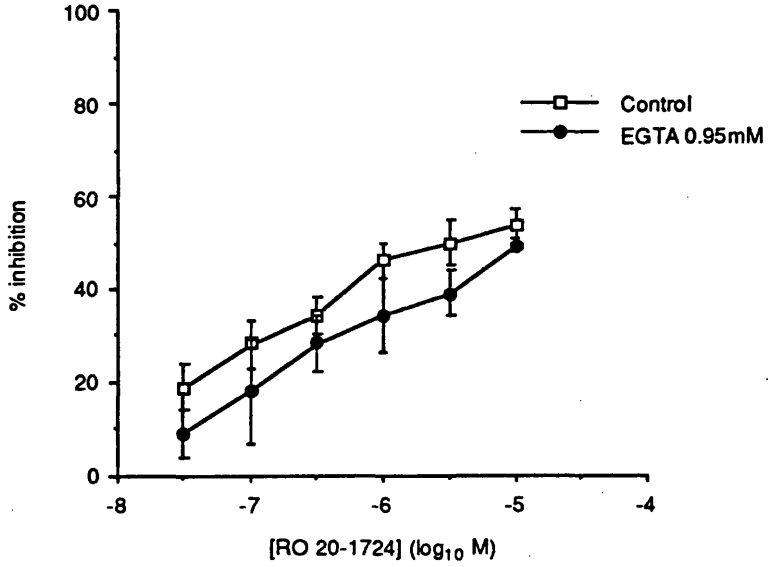


Fig 4.7 Effect of divalent cation chelators EDTA and EGTA on fMLP-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with cytochalasin B (5μg ml^{-1})+/-EDTA (1mM) or EGTA (0.95mM) prior to stimulation with fMLP (10^{-9}-10^{-6}M, 5 min, 37°C). Results shown are mean±s.e.m superoxide anion generation (nmol O_2^- 10^6 neutrophils^{-1}) of 3 separate experiments performed in duplicate.

Fig 4.8 PGE_2 (upper panel), rolipram (middle panel) and RO 20-1724 (lower panel)-mediated inhibition of fMLP (10^{-7}M)-stimulated superoxide generation by human neutrophils in the absence or presence of EGTA. Neutrophils (10^6 ml^{-1}) were incubated with PGE_2, rolipram or RO 20-1724 (5 min, 37°C) in the presence of cytochalasin B (5μg ml^{-1}) +/- EGTA (0.95mM) prior to stimulation with fMLP (5 min, 37°C). Results shown are mean±s.e.m % inhibition of the respective fMLP responses i.e.+/- EGTA of 3 separate experiments performed in duplicate.


120
100
80
60
40
20
0
% inhibition
-10
-9
-8
-7
-6
-5
-4
[PGE2] (log10 M)
Control
EGTA 0.95mM


100
80
60
40
20
0
-20
% inhibition
-10
-9
-8
-7
-6
-5
[Rolipram] (log10 M)
Control
EGTA 0.95mM


100
80
60
40
20
0
% inhibition
-8
-7
-6
-5
-4
[RO 20-1724] (log10 M)
Control
EGTA 0.95mM

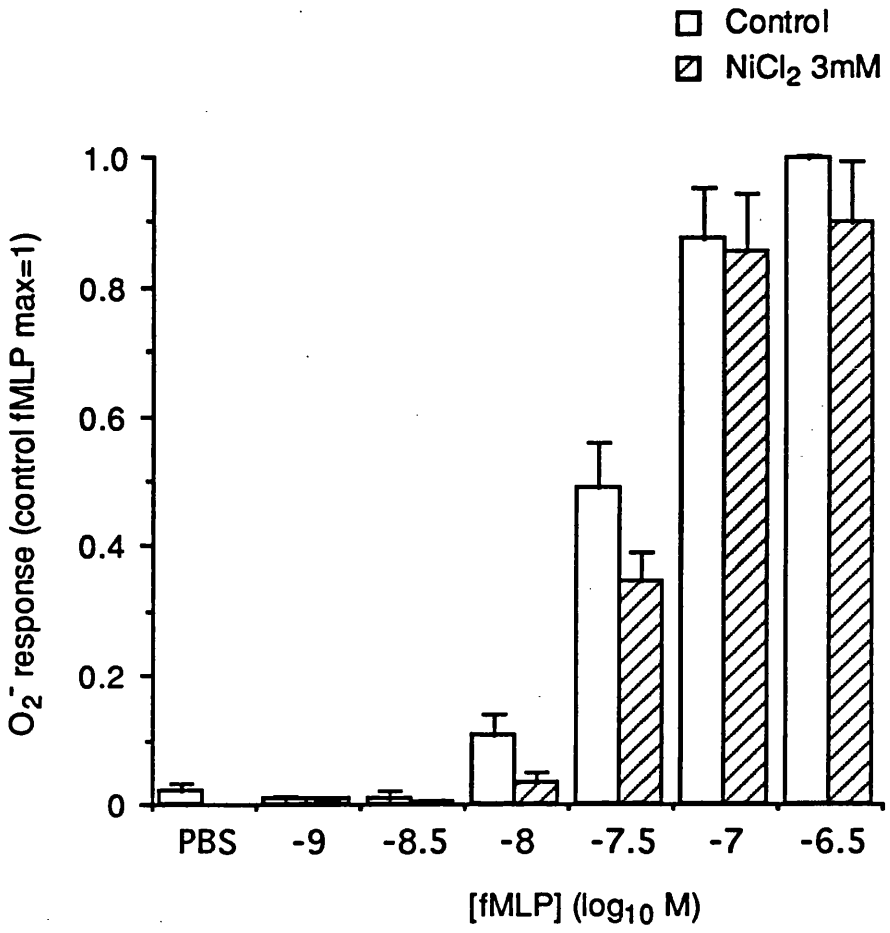


Fig 4.9 Effect of extracellular Ni^{2+} ions on fMLP-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with $NiCl_2$ (3mM) and cytochalasin B (5µg ml^{-1}) for 5 min (37°C) prior to fMLP stimulation (5min, 37°C). Results shown are mean±s.e.m fMLP responses normalised with respect to the control fMLP maximum response (=1) in the absence of $NiCl_2$ in 3 separate experiments performed in duplicate.

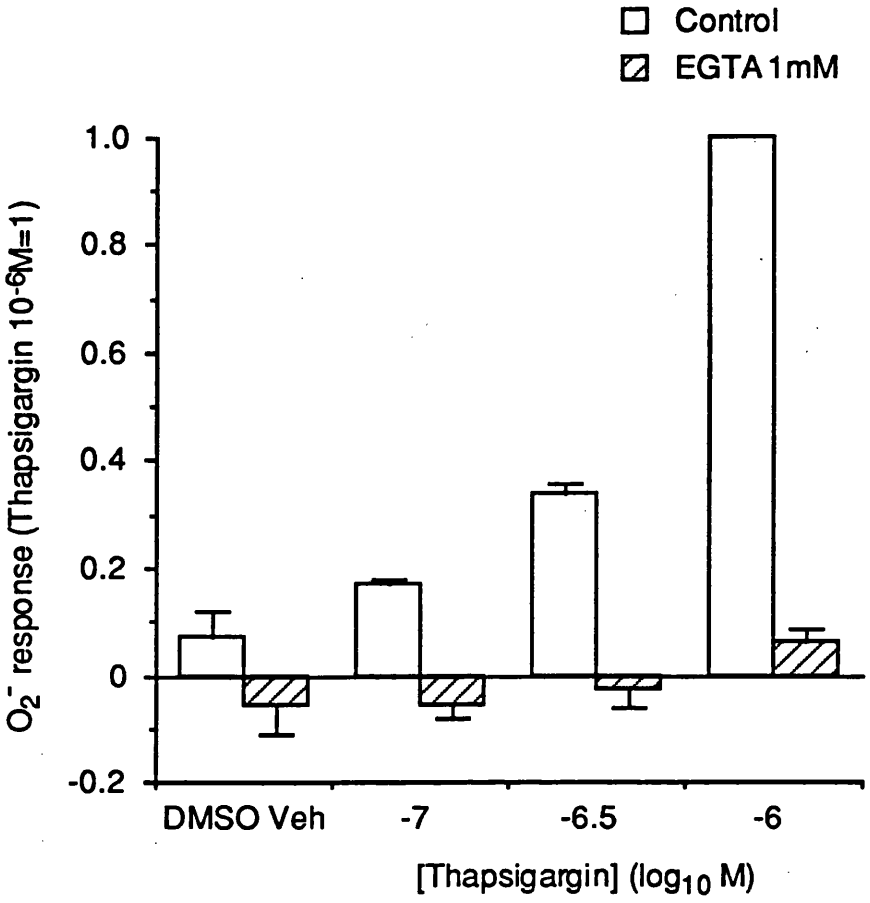


Fig 4.10 Thapsigargin-stimulated superoxide generation by human neutrophils - effect of EGTA (0.95mM). Neutrophils (10^6 ml^{-1}) were preincubated with EGTA for 5 min (37°C) prior to stimulation with thapsigargin (10 min, 37°C). Results shown are the mean±s.e.m of superoxide responses normalised with respect to the control 10^{-6}M thapsigargin response (=1) of 3 separate experiments performed in duplicate.

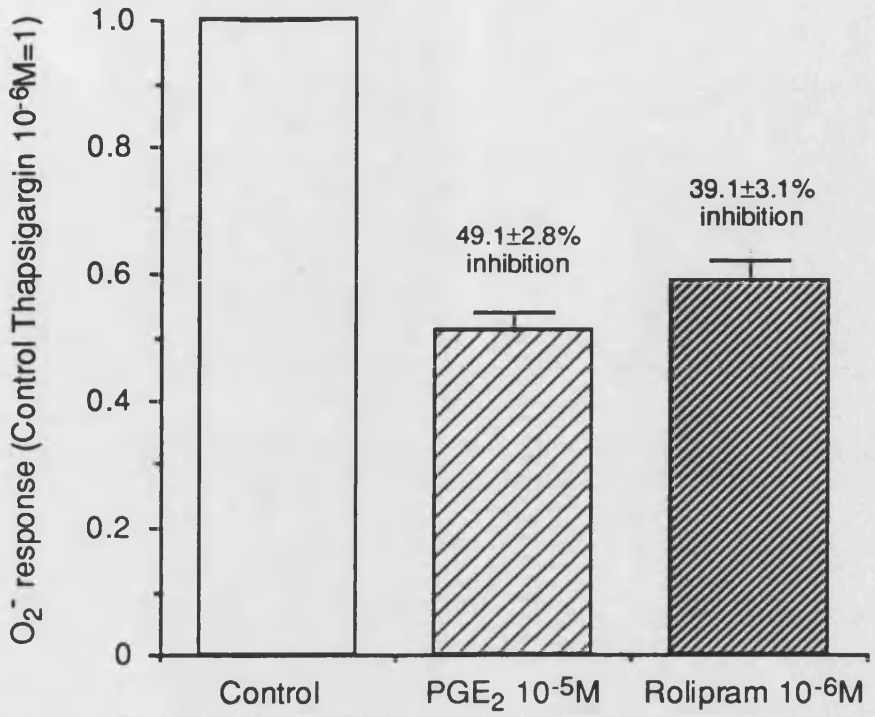


Fig 4.11 Effect of PGE_2 (10^{-5}M) and rolipram (10^{-6}M) on thapsigargin (10^{-6}M)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) preincubated with rolipram or PGE_2 for 5 min (37°C) prior to stimulation with thapsigargin (10 min, 37°C). Results shown are mean±s.e.m superoxide response normalised with respect to the thapsigargin control of 4 (PGE_2) and 3 (rolipram) separate experiments performed in duplicate.

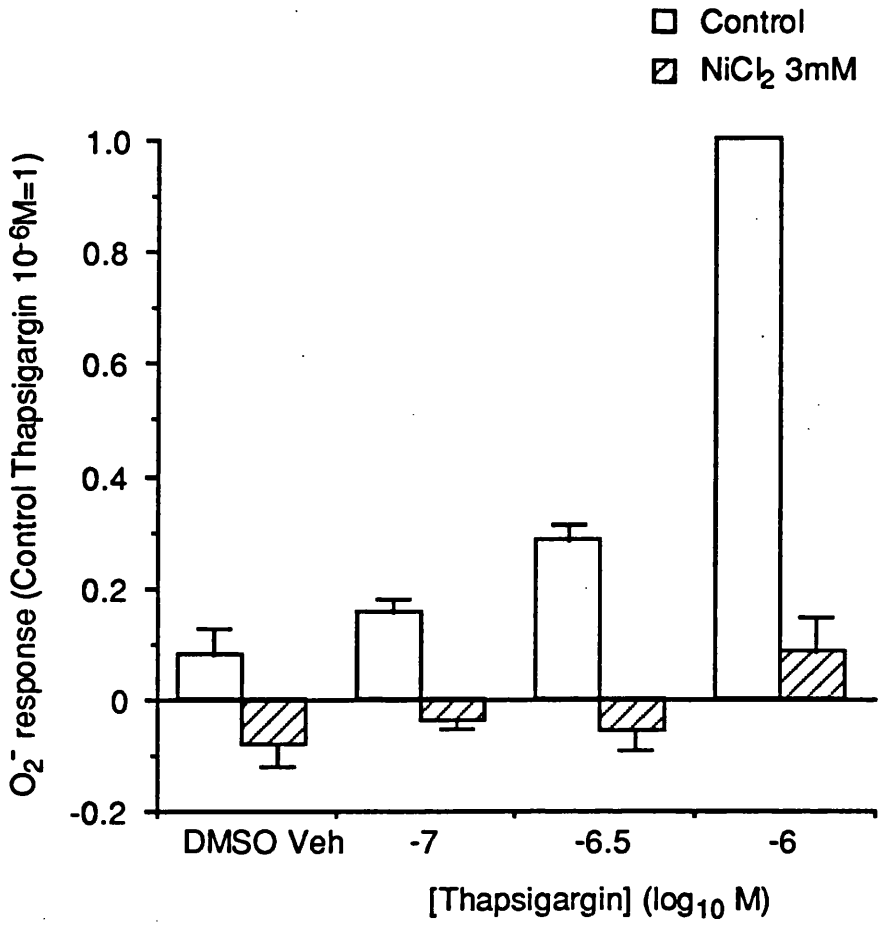


Fig 4.12 Effect of extracellular Ni^{2+} ions on thapsigargin-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with $NiCl_2$ (3mM) for 5 min (37°C) prior to thapsigargin stimulation (10min, 37°C). Results shown are mean±s.e.m thapsigargin responses normalised with respect to the control thapsigargin maximum response (=1) of 3 separate experiments performed in duplicate.

4.2.5 Effect of cAMP elevation on fMLP and thapsigargin stimulated increases in human neutrophil $[Ca^{2+}]_i$

4.2.5.1 fMLP-stimulated increases in $[Ca^{2+}]_i$ in human neutrophils

The basal $[Ca^{2+}]_i$ of the neutrophils was 32.9±1.6nM (n=6) in the presence of 1mM $CaCl_2$. fMLP stimulated a biphasic increase in $[Ca^{2+}]_i$, characterized by a rapid 'transient' increase in $[Ca^{2+}]_i$ of 207±80.9nM at 10^{-8}M (n=3) and 214.6±30.7nM at 10^{-7}M (n=6) respectively (peaking within 5 sec) which declined rapidly, followed by a protracted 'second phase' of elevated $[Ca^{2+}]_i$ with a lower plateau (Fig 4.13). The second phase remained above baseline even 5 min after stimulation.

4.2.5.2 Effect of rolipram and PGE_2 on fMLP-stimulated increase in $[Ca^{2+}]_i$

Maximally effective concentrations of PGE_2 (10^{-5}M) and rolipram (10^{-6}M) against fMLP (10^{-7}M)-stimulated O_2^- generation had no effect on the peak increase in $[Ca^{2+}]_i$ ('first phase') stimulated by fMLP (10^{-7}M), which were 96.0±3.0% (n=6) and 95.3±5.8% (n=4) respectively of the control fMLP peak $[Ca^{2+}]_i$. Both PGE_2 (10^{-5}M) (Fig 4.14) and rolipram (10^{-8}-10^{-6}M) (Fig 4.15) reduced the duration of the 'second phase'. Inhibition of fMLP-stimulated increase in $[Ca^{2+}]_i$ 30s after fMLP addition was 53±4% for PGE_2 (10^{-5}M, n=7), and for rolipram 17±10% (10^{-8}M), 41±12% (10^{-7}M) and 42±12% (10^{-6}M) (n=3 for each).

4.2.5.3 fMLP-stimulated increases in $[Ca^{2+}]_i$ using a Ca^{2+}-re-addition protocol

The Ca^{2+}-re-addition protocol was used to dissociate Ca^{2+} release from intracellular stores and Ca^{2+} influx. Extracellular Ca^{2+} ions were removed so that the increase in $[Ca^{2+}]_i$ reflected release from intracellular stores. Ca^{2+} influx could also be studied independently in the same preparation of cells by the re-introduction of extracellular Ca^{2+}

ions. The basal $[Ca^{2+}]_i$ levels were lower in the presence of 100μM $CaCl_2$ and 1mM EGTA but there was no difference in the onset time of the fMLP-stimulated 'transient'. The response declined rapidly to a new baseline, the $[Ca^{2+}]_i$ being approximately equal to that in the presence of 1mM $CaCl_2$. Re-addition of 2mM $CaCl_2$ (110s after fMLP stimulation) produced an immediate transient increase in $[Ca^{2+}]_i$ which peaked approximately 130s after fMLP stimulation and slowly returned to basal $[Ca^{2+}]_i$ levels (control basal in the presence of 1mM $CaCl_2$). In unstimulated cells, there was a similar but more transient and smaller increase in $[Ca^{2+}]_i$ on re-addition of 2mM $CaCl_2$ (Fig 4.16 and 4.17), which also returned to control basal $[Ca^{2+}]_i$ levels.

4.2.5.4 Effect of rolipram and PGE_2 on fMLP-stimulated increase in $[Ca^{2+}]_i$ using the Ca^{2+} re-addition protocol

Neither PGE_2 (10^{-5}M) and rolipram (10^{-6}M) had any effect on the rate of onset or the peak (the magnitude) of the fMLP (10^{-7}M)-stimulated Ca^{2+} 'transient'. However, there was a more rapid rate of decay of this transient in PGE_2 or rolipram pre-treated neutrophils (n=7) (Fig 4.16). In addition, PGE_2 (10^{-5}M) or rolipram (10^{-6}M) both inhibited the magnitude of the increase in $[Ca^{2+}]_i$ on re-addition of 2mM $CaCl_2$ in fMLP-stimulated neutrophils by 78±2% and 70±5% respectively (measured at fMLP T+130s, n=3 for both) (Fig 4.16). The increase in $[Ca^{2+}]_i$ on re-addition of $CaCl_2$ in unstimulated neutrophils was unaffected by these agents (Fig 4.17).

4.2.5.5 Effect of rolipram and PGE_2 on thapsigargin-stimulated increase in $[Ca^{2+}]_i$ using the Ca^{2+} re-addition protocol

Thapsigargin (10^{-6}M) stimulated an increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ions, but unlike fMLP, the level did not return to basal levels (Fig 4.18). Re-addition of 2mM $CaCl_2$ 160s after thapsigargin addition resulted in a further larger

increase in $[Ca^{2+}]_i$. This second phase was larger than that observed with fMLP (10^{-7}M), and did not return to basal levels. No consistent inhibition of this second phase of increase in $[Ca^{2+}]_i$ by rolipram (10^{-6}M) or PGE_2 (10^{-5}M) pre-treatment was observed (Fig 4.18).

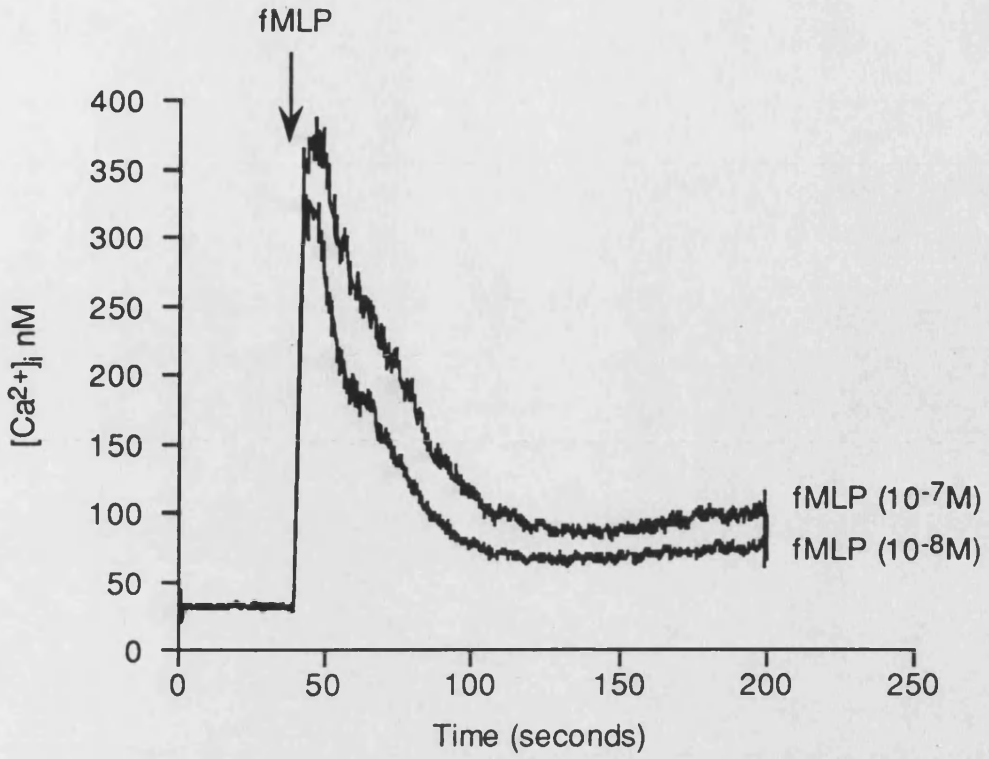


Fig 4.13 fMLP (10^{-8}M and 10^{-7}M) stimulated $[Ca^{2+}]_i$ elevation in human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were stimulated with fMLP in the presence of 1mM $MgCl_2$ and 1mM $CaCl_2$. The trace shown is taken from a single experiment but is representative of 2 other experiments.

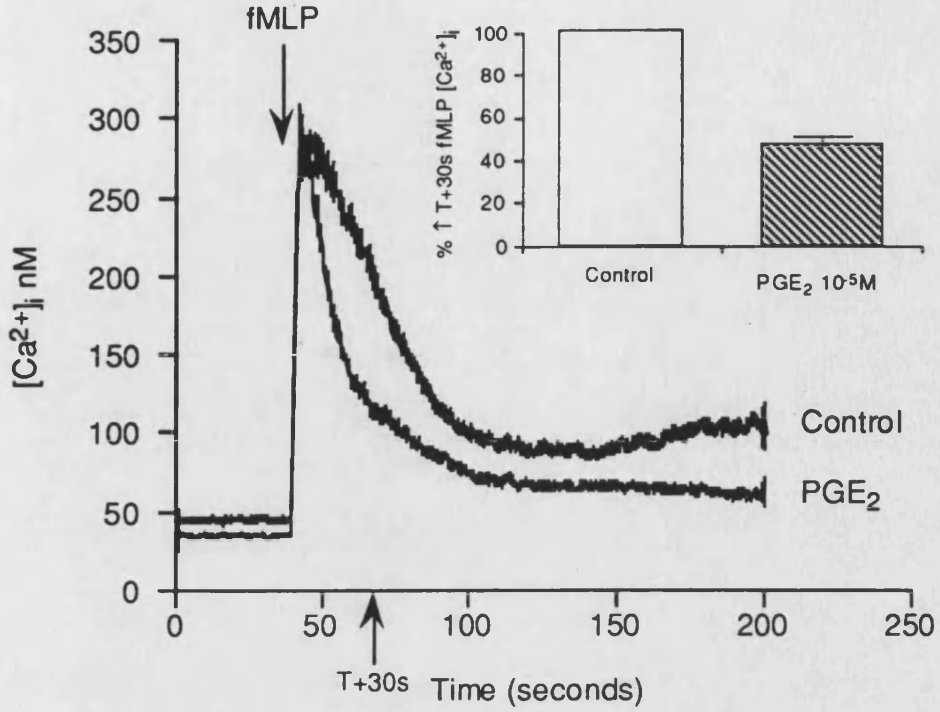


Fig 4.14 Effect of PGE_2 (10^{-5}M) of fMLP (10^{-7}M)-stimulated $[Ca^{2+}]_i$ elevation in human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were preincubated with PGE_2 for 5 min (37°C) prior to stimulation with fMLP (indicated by the arrow). The traces shown are from a single experiment but are representative of 7 other experiments. The inset shows the effect of PGE_2 on fMLP-stimulated increase in $[Ca^{2+}]_i$ measured 30s after stimulation with fMLP (T+30s) indicated by an arrow on the ordinate axis of the trace. Results are shown are the mean±s.e.m increase in $[Ca^{2+}]_i$ compared to the control fMLP response of 7 separate experiments performed in duplicate.

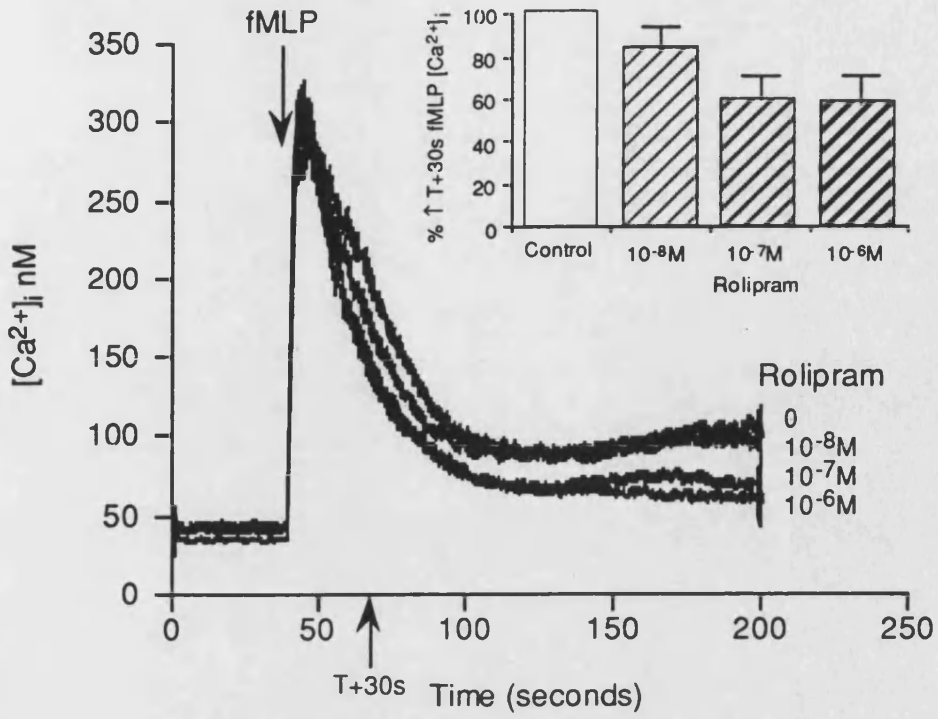
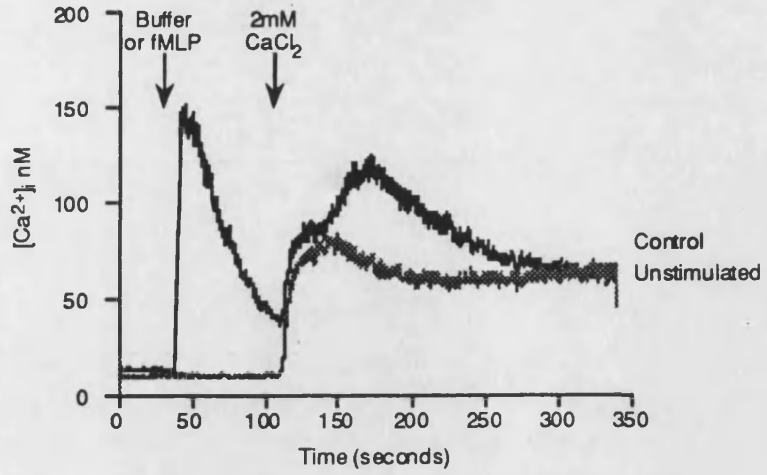
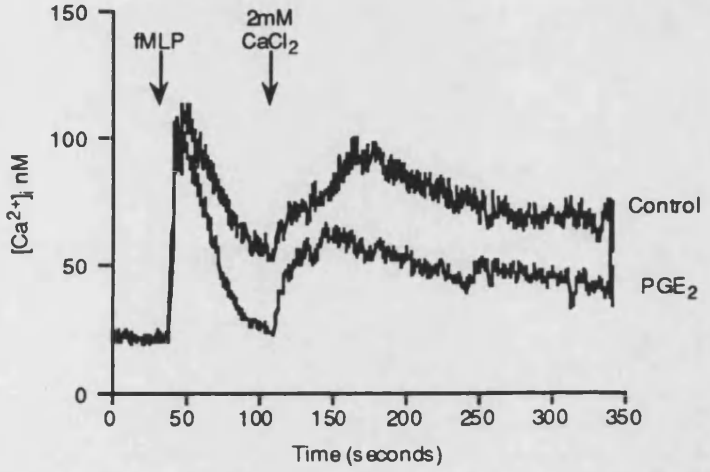
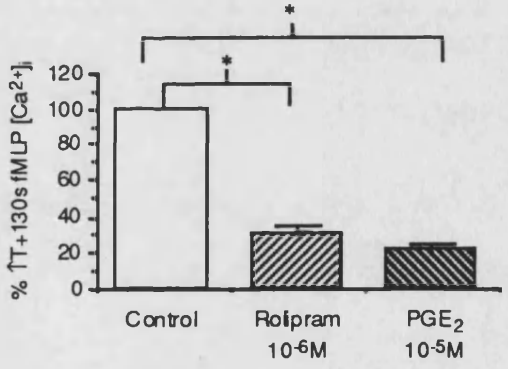


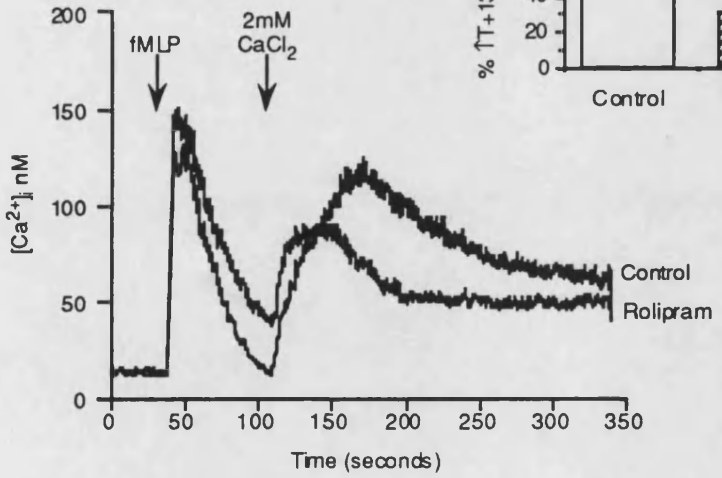
Fig 4.15 Effect of rolipram (10^{-8}-10^{-6}M) of fMLP (10^{-7}M)-stimulated $[Ca^{2+}]_i$ elevation in human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were preincubated with rolipram for 5 min (37°C) prior to stimulation with fMLP (indicated by the arrow). The traces shown are from a single experiment but are representative of at least 2 other experiments The inset shows the effect of rolipram on fMLP-stimulated increase in $[Ca^{2+}]_i$ measured 30s after stimulation with fMLP (T+30s) indicated by an arrow on the ordinate axis of the trace. Results are shown are the mean±s.e.m increase in $[Ca^{2+}]_i$ compared to the control fMLP response of 3 separate experiments performed in duplicate.

Fig 4.16 Effect of PGE_2 ($10^{-5}M$) (middle panel) and rolipram ($10^{-6}M$) (lower panel) on fMLP ($10^{-7}M$)-stimulated elevation of $[Ca^{2+}]_i$ in human neutrophils using the 're-introduction of extracellular Ca^{2+}' protocol. Upper panel shows the effect of re-introducing extracellular Ca^{2+} on unstimulated and fMLP-stimulated human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with EGTA (1mM) in the presence of 0.1mM $CaCl_2$, 1mM $MgCl_2$ and either PGE_2 or rolipram for 5 min (37°C) prior to the addition of fMLP (or buffer), followed 110s later by the re-introduction of 2mM $CaCl_2$. The inset shows the effect of rolipram and PGE_2 on the fMLP-stimulated increase in $[Ca^{2+}]_i$ on re-introduction of 2mM $CaCl_2$.


Buffer or fMLP
2mM $CaCl_2$
$[Ca^{2+}]_i$ nM
Control
Unstimulated
Time (seconds)


fMLP
2mM $CaCl_2$
$[Ca^{2+}]_i$ nM
Control
PGE_2
Time (seconds)


% ↑T+130s fMLP $[Ca^{2+}]_i$
Control
Rolipram 10^{-6}M
PGE_2 10^{-5}M


fMLP
2mM $CaCl_2$
$[Ca^{2+}]_i$ nM
Control
Rolipram
Time (seconds)

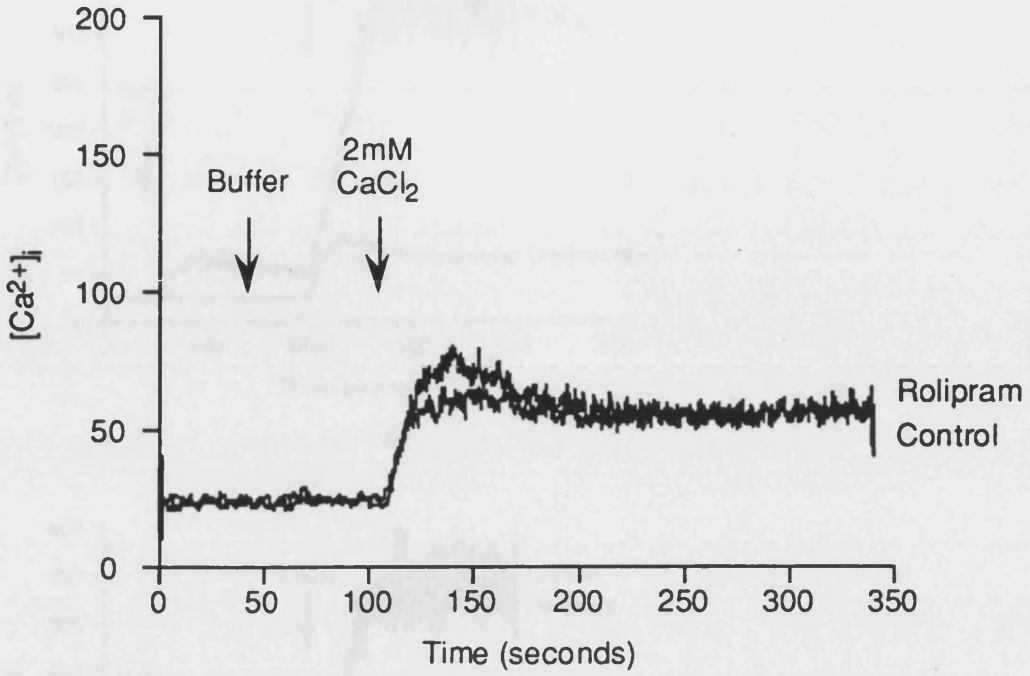


Fig 4.17 Lack of effect of rolipram (10^{-6}M) on the $[Ca^{2+}]_i$ elevation in unstimulated human neutrophils using the 're-introduction of extracellular Ca^{2+}' protocol. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with EGTA (1mM) in the presence of 0.1mM $CaCl_2$, 1mM $MgCl_2$ and rolipram for 5 min (37°C) prior to the addition of buffer, followed 110s later by the re-introduction of 2mM $CaCl_2$. The traces shown are from a single experiment but are representative of 2 other experiments.

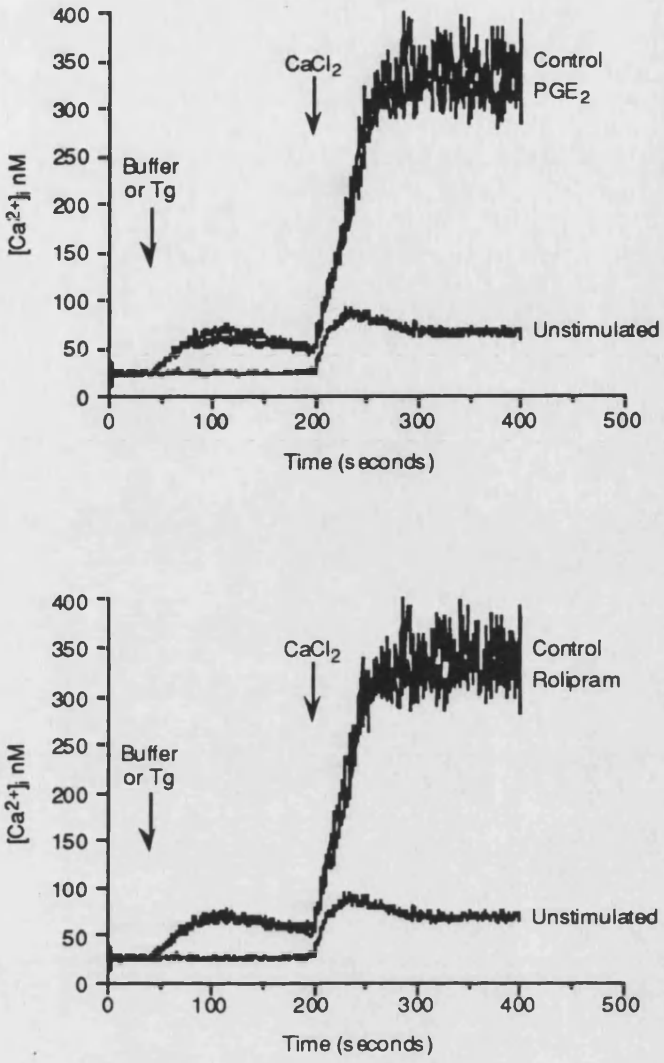


Fig 4.18 Effect of PGE_2 (10^{-5}M) (upper panel) and rolipram (10^{-6}M) (lower panel) on thapsigargin (10^{-6}M)-stimulated elevation of $[Ca^{2+}]_i$ in human neutrophils using the 're-introduction of extracellular Ca^{2+}' protocol. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with EGTA (1mM) in the presence of 0.1mM $CaCl_2$, 1mM $MgCl_2$ and either PGE_2 or rolipram for 5 min (37°C) prior to the addition of thapsigargin, followed 140s later by the re-introduction of 2mM $CaCl_2$. The traces shown are from a single experiment but are representative of 2 other experiments.

4.2.6 Effect of cAMP-elevation on fMLP-stimulated Mn^{2+} influx as a measure of Ca^{2+} influx

The effect of cAMP-elevation on fMLP-stimulated Ca^{2+} influx in human neutrophils was further investigated by measuring the influx of the Ca^{2+} surrogate, Mn^{2+} ion. The Mn^{2+} ion, unlike Ca^{2+} is not a substrate for Ca^{2+} pumps and is therefore not subject to efflux. Measuring Mn^{2+} quenching of intracellular fura-2 (λ_{ex} 360nm) therefore represented ion movement into the cell, allowing the discrimination between Ca^{2+} mobilisation and influx (Merritt *et al.*, 1989).

4.2.6.1 Effect of $MnCl_2$ concentration on fMLP-stimulated Mn^{2+} influx

In initial experiments using extracellular Mn^{2+} at 1mM, there was a basal leakage of Mn^{2+} ions into the neutrophils as observed by the quenching of fura-2 in unstimulated neutrophil. fMLP (10^{-7}M) stimulation induced further Mn^{2+} influx; but pretreating the neutrophils with PGE_2 (10^{-5}M) or rolipram (10^{-6}M) had no effect on the rate or degree of fMLP-stimulated Mn^{2+} influx (107±14% and 107±4% respectively compared to the control fMLP response 100±8%, n=3 for both).

The insensitivity of fMLP-stimulated Mn^{2+} influx to cAMP-elevating agents could be interpreted in three ways. Firstly, Mn^{2+} ion and Ca^{2+} ion influx may be differentially regulated by cAMP, or possibly elevation of cAMP does not modulate divalent cation influx, but selectively potentiates the removal of cytosolic $[Ca^{2+}]_i$.

To exclude the possibility that the Mn^{2+} concentration used (1mM) was excessive and masking inhibition of stimulated Mn^{2+} influx, the effect of adding lower concentrations of $MnCl_2$ to the extracellular medium was investigated.

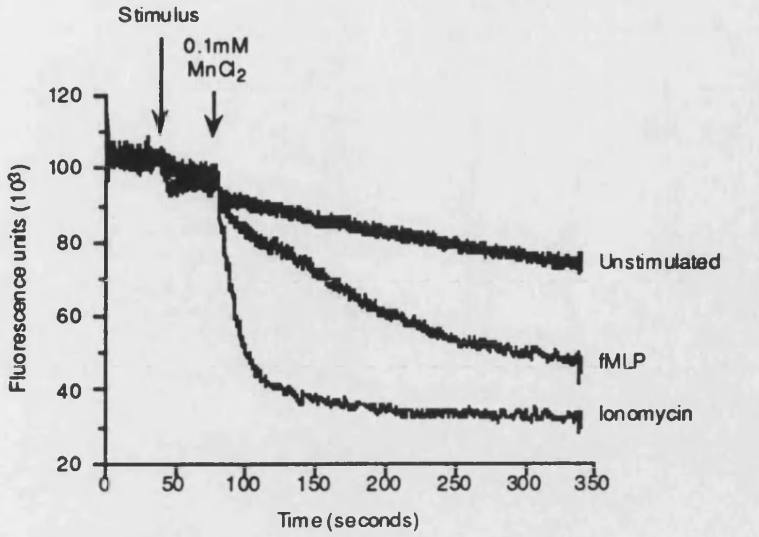
Reducing the extracellular Mn^{2+} concentration from 1mM to 0.3mM made no difference to Mn^{2+} leakage into unstimulated cells, nor was there a change in Mn^{2+} influx into fMLP

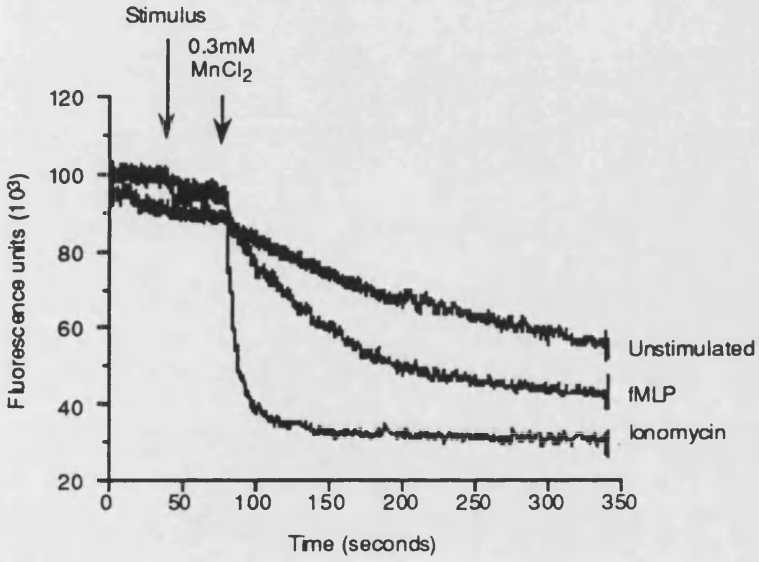
(10^{-7}M) or ionomycin ($2x10^{-6}$M)-stimulated neutrophils (Fig 4.19). Lowering the $MnCl_2$ concentration to 0.1mM, substantially reduced the leakage of Mn^{2+} ions into unstimulated cells compared to that in the presence of 1mM $MnCl_2$. In contrast, fMLP (10^{-7}M) and ionomycin ($2x10^{-6}$M) stimulated Mn^{2+} influx was identical to that observed in the presence of 1mM $MnCl_2$ (Fig 4.19).

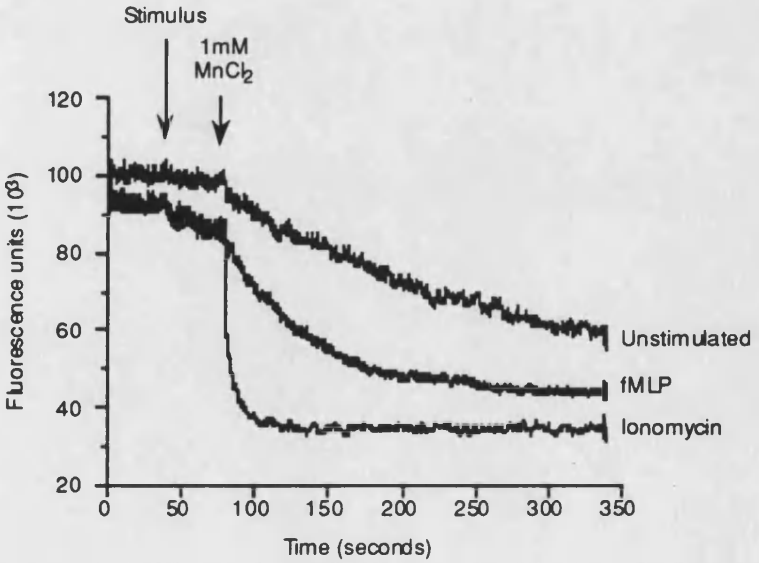
4.2.6.2 Effect of rolipram and PGE_2 on fMLP-stimulated Mn^{2+} (0.1mM) influx

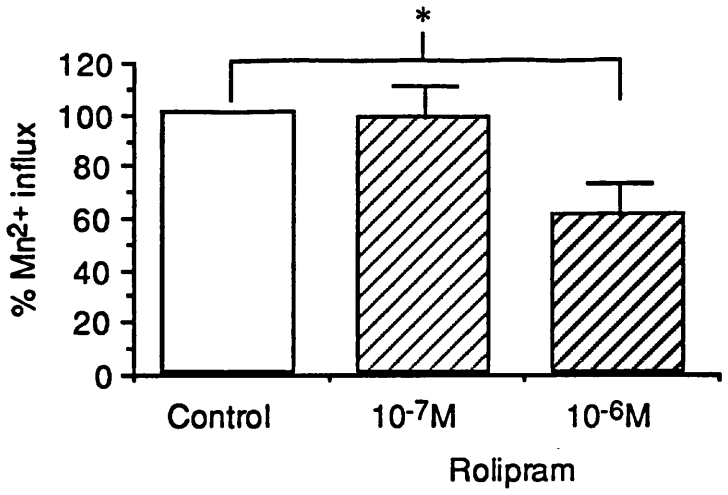
fMLP (10^{-7}M)-stimulated Mn^{2+} influx (0.1mM) into human neutrophils was concentration-dependently inhibited by pretreating the neutrophils with rolipram (10^{-7}-10^{-6}M) or PGE_2 (10^{-6}-10^{-5}M) (Fig 4.20) (n=3). The degree of inhibition of fMLP-stimulated Mn^{2+} influx observed between experiments was variable, but rolipram was consistently more potent and effective than PGE_2 (n=4 donors). However the combination of maximally effective concentrations of PGE_2 (10^{-5}M) and rolipram (10^{-6}M) resulted in an additive inhibition of fMLP (10^{-7}M)-stimulated Mn^{2+} (0.1mM) influx (Fig 4.21), which was greater than either agent alone (n=4), but did not inhibit fMLP-stimulated Mn^{2+} (1mM) influx (n=3) (fig 4.22).

Fig 4.19 Effect of $MnCl_2$ concentration on Mn^{2+} influx in unstimulated and fMLP (10^{-7}M) or ionomycin ($2x10^{-6}$M)-stimulated human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with 1mM $MgCl_2$, 0.1mM $CaCl_2$ for 5 min (37°C) prior to the addition of buffer, fMLP or ionomycin ($2x10^{-6}$M) followed by the introduction of $MnCl_2$ (0.1, 0.3 or 1mM) 40s later. The traces shown are from a single experiment but are representative of 2 other experiments.


Stimulus
0.1mM
$MnCl_2$
Fluorescence units (10^3)
120
100
80
60
40
20
0
50
100
150
200
250
300
350
Time (seconds)
Unstimulated
fMLP
Ionomycin


Stimulus
0.3mM
$MnCl_2$
Fluorescence units (10^3)
120
100
80
60
40
20
0
50
100
150
200
250
300
350
Time (seconds)
Unstimulated
fMLP
Ionomycin


Stimulus
1mM
$MnCl_2$
Fluorescence units (10^3)
120
100
80
60
40
20
0
50
100
150
200
250
300
350
Time (seconds)
Unstimulated
fMLP
Ionomycin



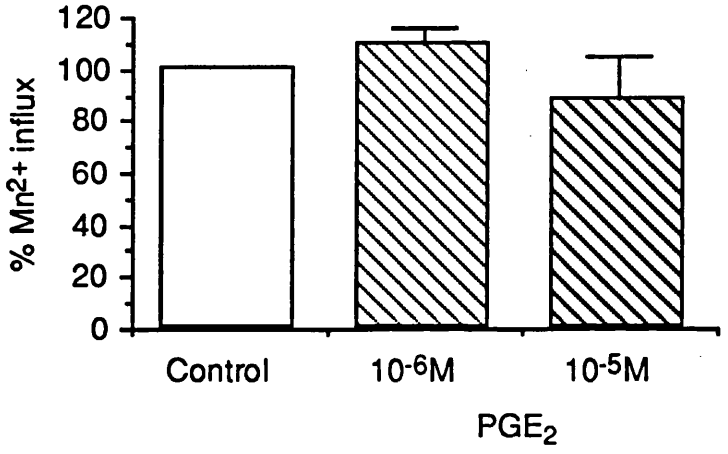


Fig 4.20 Effect of PGE_2 (10^{-6}-10^{-5}M) and rolipram (10^{-7}-10^{-6}M) on fMLP (10^{-7}M)-stimulated Mn^{2+} (0.1mM) influx in human neutrophils (upper panel). Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with buffer, PGE_2 or rolipram for 5 min (37°C) in the presence of 1mM $MgCl_2$, 0.1mM $CaCl_2$ prior to the addition of buffer, fMLP or ionomycin ($2x10^{-6}$M, positive control) followed by the introduction of $MnCl_2$ (0.1mM) 40s later. Results shown are the effect of PGE_2 and rolipram on fMLP-stimulated Mn^{2+} influx as mean±s.e.m % of the control fMLP response quantified as AUC. Statistical significance indicated by an asterisk representing $P<0.05$ as determined by Student's t-test.

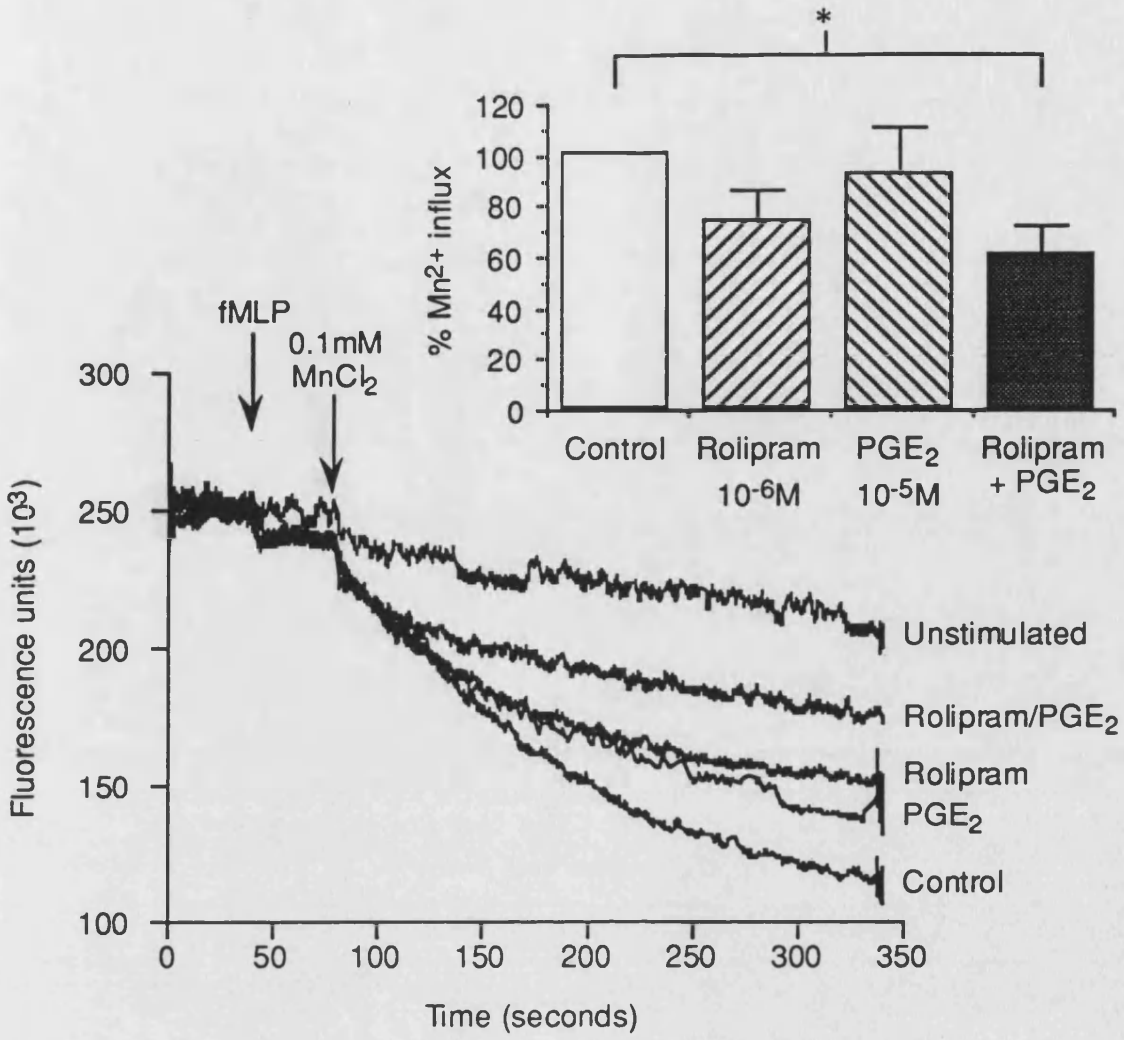


Fig 4.21 Effect of PGE_2 (10^{-5}M) and rolipram (10^{-6}M) alone and in combination on fMLP (10^{-7}M)-stimulated Mn^{2+} (0.1mM) influx in human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with PGE_2 +/- rolipram in the presence of 1mM $MgCl_2$ and 0.1mM $CaCl_2$ for 5 min (37°C) prior to the addition of buffer or fMLP, followed by the introduction of $MnCl_2$ 40s later. The traces shown are from a single experiment but are representative of 3 other experiments. The inset shows PGE_2 +/- rolipram-mediated inhibition of fMLP-stimulated Mn^{2+} (0.1mM) influx as mean±s.e.m % of the control fMLP response quantified as AUC of 4 separate experiments. Statistical significance in the inset is indicated with the asterisk representing $P<0.05$ as determined by Student's t-test.

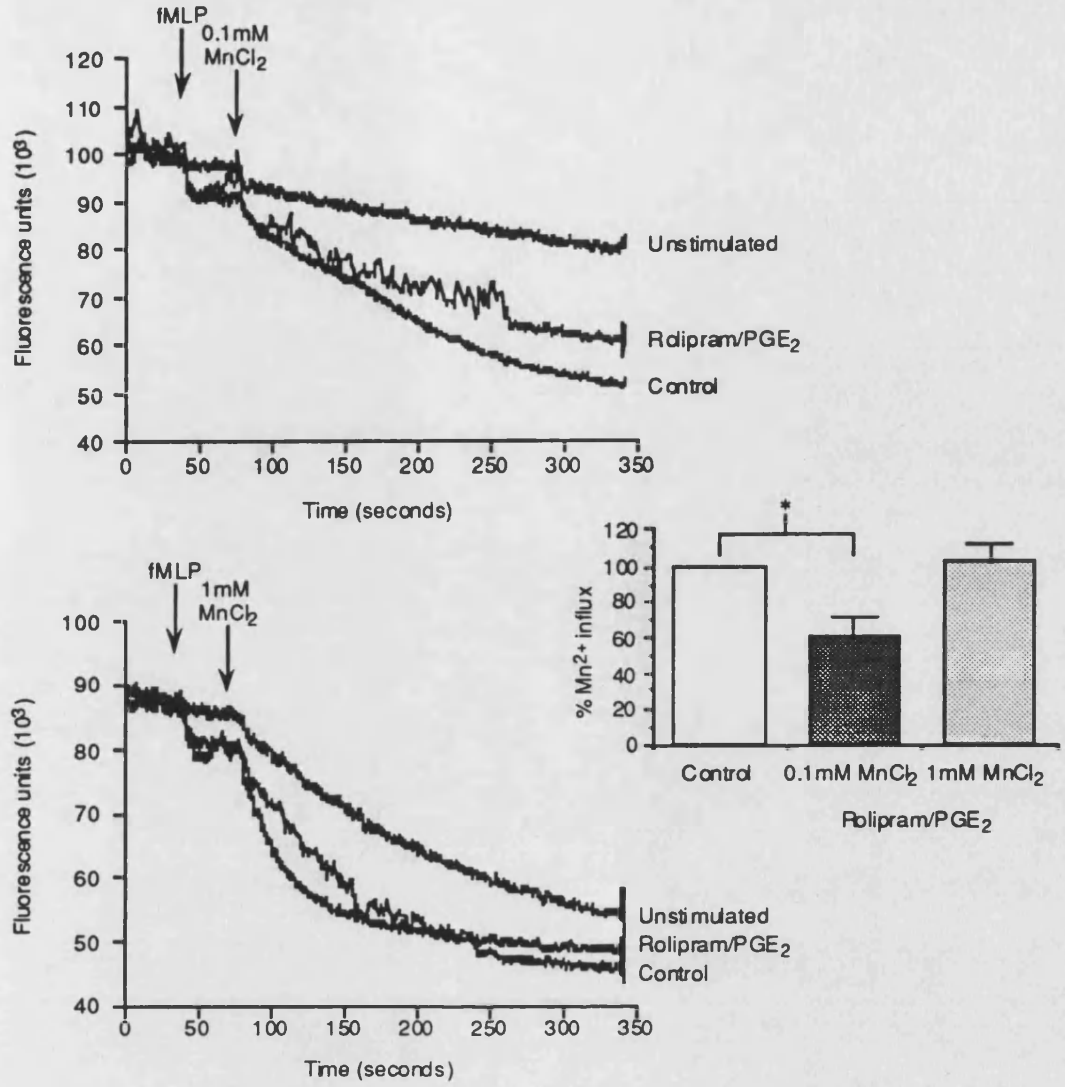


Fig 4.22 Comparison of the ability of rolipram (10^{-6}M) combined with PGE_2 (10^{-5}M) to inhibit fMLP (10^{-7}M)-stimulated Mn^{2+} (0.1mM, upper panel) and (1mM, lower panel) influx in human neutrophils. Fura-2-loaded neutrophils ($5x10^6$ ml^{-1}) were incubated with rolipram combined with PGE_2 in the presence of 1mM $MgCl_2$ and 0.1mM $CaCl_2$ for 5 min (37°C) prior to the addition of buffer or fMLP, followed by the introduction of $MnCl_2$ 40s later. The traces shown are from a single experiment but are representative of 2 other experiments. The inset shows the effect of rolipram combined with PGE_2 on fMLP-stimulated Mn^{2+} influx as mean±s.e.m % control fMLP response quantified as AUC, from the time of $MnCl_2$ addition at 80s to 340s, of 3 separate experiments. Statistical significance in the inset is indicated by an asterisk representing $P<0.05$ as determined by Student's t-test.

4.3 DISCUSSION

The data presented in this chapter suggest that:-

1. Non-selective (IBMX) and type IV-selective (rolipram) PDEIs inhibited fMLP-stimulated O_2^- generation, although they stimulated little increase in neutrophil cAMP levels. PGE_2 was a potent and effective stimulus of cAMP elevation at concentrations which inhibited fMLP-stimulated O_2^- generation in the absence or presence of PDEIs.

2. PGE_2 inhibition of fMLP-stimulated O_2^- generation was potentiated by IBMX or RO 20-1724, which suggests that cAMP elevation is a common mechanism. Further evidence for cAMP elevation as the transduction mechanism was the reversal of IBMX and PGE_2 inhibition of fMLP-stimulated O_2^- generation by the protein kinase A inhibitor H-89.

3. fMLP-stimulated O_2^- generation was partially inhibited by the chelation of extracellular Ca^{2+} ions suggesting that Ca^{2+} influx was involved in fMLP-stimulated human neutrophil activation. As rolipram and PGE_2 suppressed fMLP-induced increases in $[Ca^{2+}]_i$; inhibition of Ca^{2+} influx may account in part for their inhibitory effect on neutrophil activation.

4. Neither rolipram nor PGE_2 inhibited fMLP-induced mobilisation of Ca^{2+} ions from intracellular stores; but both agents attenuated fMLP-stimulated Ca^{2+} (and Mn^{2+}) influx.

4.3.1 Effect of PDEIs and PGE_2 on cAMP levels and superoxide generation in human neutrophils

The isoenzyme selectivity and potency order of the PDEIs tested in this study as inhibitors of fMLP-stimulated O_2^- generation suggests that the predominant phosphodiesterase activity in human neutrophils is the type IV isoform:-

*rolipram	>	*RO 20-1724	>	*IBMX	>	#milrinone
type IV		type IV		non-selective		type III

(*Wright *et al.*, 1990) (#Lindgren *et al.*, 1990)

These results are in agreement with published data on phosphodiesterase activity isolated from human neutrophils, PDEI-stimulated elevation of neutrophil cAMP levels and modulatory activity on O_2^- generation (Nielson *et al*, 1990, Ho *et al.*, 1990, Schudt *et al.*, 1991).

However, in this study, rolipram and IBMX induced little elevation of neutrophil cAMP levels in either unstimulated or fMLP-stimulated human neutrophils, at concentrations which clearly inhibited fMLP-stimulated O_2^- generation. The levels of cAMP were higher in fMLP-stimulated neutrophils pre-treated with rolipram but not with IBMX. The discrepancy between the two PDEIs on fMLP-stimulated neutrophil cAMP levels has been reported by others (Schudt *et al.*, 1991). One possibility is that phosphodiesterase inhibition reveals potentiation of adenylate cyclase activation by endogenously generated adenosine by fMLP (Iannone *et al.*, 1989). This effect of fMLP on neutrophil cAMP levels was abolished by adding adenosine deaminase to inactivate endogenous adenosine or IBMX which is an adenosine receptor antagonist (Iannone *et al.*, 1989).

Consequently, the ability of PDEIs to stimulate cAMP elevation in naive or fMLP-stimulated neutrophils is not easily reconciled with their ability to inhibit neutrophil function, in terms of potency or efficacy. The results of the PDEI experiments on cAMP-elevation and neutrophil activation described in this chapter suggest that inhibition of

fMLP-stimulated O_2^- generation is exquisitely sensitive to modulation by cAMP-dependent processes or they are working through a cAMP-unrelated mechanism. It is clear however that small or undetectable increases in total cAMP content are sufficient to inhibit the respiratory burst. Furthermore, total cellular cAMP levels may not correlate well with the inhibitory effect of cAMP-elevating agents. Measurement of localised or regional increases in cAMP may provide more accurate correlation with the observed changes in neutrophil function.

In contrast to the PDEIs, PGE_2 stimulated cAMP elevation in human neutrophils at concentrations which inhibited fMLP-stimulated O_2^- generation and the results in chapter 3 suggest that they are mediated by the same prostanoid EP receptor. Nonetheless, although PGE_2-induced cAMP elevation and inhibition of neutrophil activation may be causally related, this alone is not sufficient evidence for cAMP elevation as the signal transduction pathway via which PGE_2 modulates neutrophil function.

Forskolin, the direct activator of adenylate cyclase, did not stimulate high levels of cAMP accumulation (in the presence of IBMX) above basal compared to PGE_2, which may be related to the poor inhibitory activity of forskolin on superoxide generation observed in chapter 3.

However, on comparing the effects of PDEIs and PGE_2 alone and in combination on human neutrophil function, the findings presented in this study suggest that they share a common transducer molecule - cAMP. Most notably, PGE_2-stimulated cAMP elevation and inhibition of fMLP-stimulated O_2^- generation by human neutrophils was enhanced by the presence of PDEIs. The $p[A_{50}]$ for PGE_2-inhibition of fMLP-stimulated O_2^- generation was concentration-dependently shifted leftward by IBMX and RO 20-1724; which is evidence that PGE_2 inhibition of fMLP-stimulated O_2^- generation is mediated via cAMP elevation. IBMX also produced a leftward shift of PGA_1 inhibition of fMLP-stimulated O_2^- generation, demonstrating that this effect of PDEIs was not restricted to

PGE_2 but extended to other EP receptor agonists. A similar interaction was demonstrated by Kenakin & Beek (1984), who described IBMX potentiation of β-adrenergic agonist (isoprenaline)-induced positive ionotropy in guinea-pig papillary muscles, and producing a ten-fold increase in the isoprenaline $p[A_{50}]$.

There were also many similarities between PDEI and PGE_2 effects on human neutrophil function, for instance PGE_2-stimulated cAMP elevation was augmented by the presence of fMLP, as was rolipram. In addition, the ability of the protein kinase A inhibitor, H89, to concentration-dependently reverse both PGE_2 and IBMX inhibition of fMLP-stimulated O_2^- generation by human neutrophils also suggests that both agents act via cAMP-elevation.

The adenylate cyclase inhibitor, SQ 22356, failed to attenuate PGE_2-mediated inhibition of fMLP-stimulated O_2^- generation. However, SQ 22356 attenuated IBMX-mediated inhibition of fMLP-stimulated O_2^- generation in two experiments, contrary to the reported adenylate cyclase inhibitory activity of this compound at the same concentration (10^{-4}M) on PGE_2-mediated inhibition of fMLP-stimulated O_2^- generation in human neutrophils reported by Talpain *et al.* (1994). It is unclear why there is a discrepancy between the effects of SQ 23356 described in the present study and that of Talpain *et al.*, other than a difference in preincubation time for SQ 22356 with the neutrophils prior to addition of PGE_2. A 20 min preincubation time was used in the present study whilst Talpain & Armstrong used 2 min, but considering the requirement for SQ 22356 to enter the cells to reach the site of action, adenylate cyclase, the longer preincubation period would have been more likely to ensure optimum equilibration time.

4.3.2 Effect of rolipram and PGE_2 on fMLP-stimulated increases in neutrophil $[Ca^{2+}]_i$

The results in this study showed that fMLP-stimulated O_2^- generation was partially reduced by chelation of extracellular Ca^{2+} ions; implying that fMLP-mediated neutrophil activation involved Ca^{2+} influx. Indeed, other functions such as fMLP-stimulated human neutrophil chemotaxis and adhesion, are suppressed in the presence of EGTA (Merritt *et al.*, 1991 and Davies *et al.*, 1990, respectively). In addition to this, fMLP-stimulated neutrophil degranulation and O_2^- generation were completely abolished by simultaneously depleting the intracellular Ca^{2+} stores and buffering $[Ca^{2+}]_i$ with intracellular Ca^{2+} ion chelators such as BAPTA (O'Flaherty *et al.*, 1991); demonstrating that increases in $[Ca^{2+}]_i$ were required for fMLP-activation of neutrophils. However, the same study showed that Ca^{2+}-depleted human neutrophils were still able to degranulate and generate O_2^- anions albeit at a lower level, on stimulation with fMLP in spite of the absence of an increase in $[Ca^{2+}]_i$.

The preliminary experiments in this study measuring fMLP-stimulated increases in $[Ca^{2+}]_i$ found that the response was biphasic with the characteristics described in other publications in the literature (Sage *et al.*, 1990, Demaurex *et al.*, 1992). Their studies showed that fMLP stimulated increase in $[Ca^{2+}]_i$ involved the release of Ca^{2+} ions from intracellular stores and the opening of plasma membrane Ca^{2+} channels allowing the influx of extracellular Ca^{2+} ions.

In the present study, rolipram or PGE_2, at concentrations which were maximally effective against fMLP (10^{-7}M)-stimulated O_2^- generation, modulated fMLP-stimulated increase in $[Ca^{2+}]_i$ in human neutrophils. As neither agent had any effect on the onset or the size of the first phase of the increase in $[Ca^{2+}]_i$, the 'Ca^{2+} transient'; inhibition of the mobilisation of intracellular Ca^{2+} stores seemed an unlikely mechanism of action (Schudt

et al., 1991 and Hecker *et al.*, 1990 respectively). Furthermore, other cAMP-elevating agents such as PGD_2 and the cell-permeable cAMP analogue, dibutyryl cAMP, also have no effect on the fMLP-stimulated 'Ca^{2+} transient' (Simpkins *et al.*, 1990, Ney & Schrör, 1991).

However, in the present study, the duration and amplitude of the 'second phase' (resulting from Ca^{2+} influx - see introduction) of the fMLP-stimulated increase in $[Ca^{2+}]_i$ in human neutrophils pre-treated with either rolipram or PGE_2 was reduced. The selective diminution of this protracted increase in $[Ca^{2+}]_i$ by PDEIs and PGE_2 is similar to that noted by Schudt *et al.* (1991) and Hecker *et al.* (1990) respectively. These data suggest that cAMP-elevation reduced $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx and/or promoting Ca^{2+} sequestration.

The Ca^{2+} re-addition protocol was adopted to temporally dissociate the two phases of increase in $[Ca^{2+}]_i$; and enabled the study of rolipram and PGE_2 on fMLP-stimulated Ca^{2+} mobilisation independently of Ca^{2+} influx and vice versa. As indicated earlier in this discussion, in the absence of extracellular Ca^{2+} ions, rolipram or PGE_2 did not delay the onset of Ca^{2+} mobilisation nor did they suppress the peak increase in $[Ca^{2+}]_i$. However, both agents accelerated the decay of this 'Ca^{2+} transient' suggesting that cAMP-elevation may increase the rate of removal of cytosolic free Ca^{2+} ions.

In the same neutrophil preparation, the re-introduction of Ca^{2+} ions after the mobilisation-dependent increase in $[Ca^{2+}]_i$ returned to basal levels resulted in an additional influx-dependent increase in $[Ca^{2+}]_i$. Both rolipram and PGE_2 reduced the magnitude of the fMLP-stimulated influx-dependent increase in $[Ca^{2+}]_i$ to almost unstimulated levels. Although the Ca^{2+} entry induced increase in $[Ca^{2+}]_i$ was almost completely abolished by rolipram or PGE_2; it was unclear from these Ca^{2+} re-addition experiments whether this was achieved by inhibiting Ca^{2+} channel opening or promoting Ca^{2+} sequestration and/or

efflux.

PDEIs exhibited a similar inhibitory effect on fMLP-stimulated increases in $[Ca^{2+}]_i$ in fura-2 loaded human neutrophils in the presence of thimerosal (Schudt *et al.*, 1991) which prolonged and increased the magnitude of the second phase of fMLP-stimulated increase in $[Ca^{2+}]_i$. Although they observed inhibition of this influx component by PDEIs, the inhibition was difficult to quantitate as the use of thimerosal did not differentiate between inhibition of Ca^{2+} influx per se and potentiation of Ca^{2+} efflux. Another complication was that the mechanism of action by which thimerosal prolonged and increased the fMLP-stimulated influx was unknown. Thimerosal itself induced a small rise in $[Ca^{2+}]_i$ (even in the absence of extracellular Ca^{2+} ions) but did not affect the function of the plasma membrane Ca^{2+} pump. These observations suggested that thimerosal may prevent repletion of the intracellular Ca^{2+} stores depleted by fMLP activation of the neutrophil (Hatzelmann *et al.*, 1990).

Hecker *et al.* (1990) used ^{45}Ca uptake as an index of Ca^{2+} influx, and showed that PGE_2 inhibited fMLP-stimulated ^{45}Ca uptake by human neutrophils. However the response was small, and the experimental protocol used made it impossible to determine whether the uptake of ^{45}Ca represented purely influx, or if there was a component of binding of ^{45}Ca to cellular protein.

Thus, many different approaches have been adopted in order to demonstrate that putative neutrophil inhibitors have a direct effect on Ca^{2+} influx as a mechanism of action but to date, there has been little progress in this area of research. However, rolipram and PGE_2 mediated inhibition of fMLP-stimulated Mn^{2+} (Ca^{2+} surrogate) influx in human neutrophils as described in this chapter are the first direct demonstrations of PDEI and PGE_2 inhibition fMLP-stimulated Ca^{2+} influx in human neutrophils. However the disparate effect of rolipram and PGE_2 on Mn^{2+} influx may be explained by a possible effect of Mn^{2+} accumulation on adenylate cyclase activation. The human neutrophil

adenylate cyclase isoform(s) have not been identified although at least 8 different mammalian isoforms have been characterized (Kruprinski *et al.*, 1992), some of which are activated by Mn^{2+} ions in the mM concentration range leading to approximately 2-fold increase in enzyme activity (Jakobs *et al.*, 1984). It is possible then that Mn^{2+} ions become concentrated within the neutrophil cytosol and may actually compete with the PGE_2-receptor complex for activation of adenylate cyclase. PGE_2 normally causes a 8-10 fold increase in human neutrophil cAMP levels; if Mn^{2+} ions reduce PGE_2-stimulated cAMP levels this would result in a reduction in the inhibitory effect of the expected degree of inhibition of Mn^{2+} influx. If this were the case, rolipram would be potentially more effective as by preventing the breakdown of cAMP stimulated by the effect of Mn^{2+} ions on adenylate cyclase potentiates the inhibitory effect of cAMP elevation on Mn^{2+} influx.

Mn^{2+} ions are not the only divalent cations which can influence adenylate cyclase activity, some isoforms of adenylate cyclase are inhibited by Ca^{2+} ions (Kruprinski *et al.*, 1992). Thus changes in $[Ca^{2+}]_i$ could also affect adenylate cyclase activation in the human neutrophil just as cAMP levels modulate $[Ca^{2+}]_i$ which suggest that these two signal transduction molecules interact intimately to regulate neutrophil function. Mn^{2+} ions may also affect the function of the type IV phosphodiesterase, as there appears to be an absolute functional requirement for divalent cations. EDTA treatment inactivates the type IV PDE and catalytic activity is only restored by the addition of Mg^{2+}, Mn^{2+} or Zn^{2+} but not Ca^{2+} ions. This effect of Mn^{2+} ions on PDE activity may affect the ability of cAMP-elevating agents to inhibit Mn^{2+} influx. Relatively high concentrations of rolipram were required to inhibit fMLP-stimulated Mn^{2+} influx compared with O_2^- generation and the second phase of increase in $[Ca^{2+}]_i$, and may be explained by the higher concentration of rolipram required to overcome the stimulatory effect of Mn^{2+} ions accumulating in the neutrophils. PGE_2-stimulated increases in cAMP are mediated by activating adenylate cyclase, however the accumulation of Mn^{2+} ions within the cell may stimulate PDE

activity attenuating the increase in cAMP and abrogating the inhibitory effects of PGE_2 on Mn^{2+} influx. The variability of the inhibition of Mn^{2+} influx by PGE_2 and rolipram observed may reside at the level of phosphodiestase activity in each neutrophil preparation. Consequently, the effect of rolipram and PGE_2 on fMLP-stimulated neutrophil Mn^{2+} influx may underestimate their modulation of Ca^{2+} influx.

The role of Ca^{2+} influx in cell activation has been questioned (Clapham, 1995) - is it just a physiological mechanism to refill intracellular stores or does it have a more direct role in cell activation? The latter is a distinct possibility as many cellular enzymes and processes occur in association with the plasma membrane. In the neutrophil this includes adenylate cyclase, the NADPH-oxidase, phosphodiesterase and obviously Ca^{2+} influx channels. Thus the main neutrophil functions and biochemical processes studied in this thesis are located at the plasma membrane and suggest that they are physically in close proximity. This would allow highly regulated interactions between these cellular processes and emphasises the complexity of neutrophil activation regulation which can occur at many levels.

The evidence in the literature suggests that in human neutrophils, fMLP-stimulated Ca^{2+} and Mn^{2+} influx have identical kinetics (Sage *et al.*, 1990) and is closely linked to the emptying of the intracellular stores (Demaurex *et al.*, 1992). Intuitively then, Ca^{2+} influx, and therefore Mn^{2+} influx, in the human neutrophil is closely associated with the filling state of the intracellular stores of Ca^{2+} ions (Demaurex *et al.*, 1992).

Nevertheless, the ion channels through which Ca^{2+} and Mn^{2+} ions permeate the plasma membrane to enter the neutrophil have not been elucidated. However, patch-clamping studies on human neutrophils have established the presence of a non-specific cation channel, which is activated by increases in $[Ca^{2+}]_i$ and is permeable to Ca^{2+} ions (von Tscharner *et al.*, 1986). However, it is unclear whether Mn^{2+} ions could permeate this channel, or if this non-specific cation channel is inhibited by rolipram and PGE_2. cAMP-

modulated ion fluxes have been reported in the neutrophil including that of Schumann *et al.*, (1992), describing dibutyryl cAMP and adenosine agonist (NECA) inhibition of a fMLP-stimulated non-specific cation current in whole cell voltage-clamped human neutrophils.

The characterization of Ca^{2+} permeable channels in the neutrophil plasma membrane has so far depended on indirect evidence such as the channel blocking activity of divalent (Ni^{2+} and Co^{2+}) and trivalent (La^{3+}) cations (Andersson *et al.*, 1986). Human neutrophils do not possess voltage-operated Ca^{2+} channels, as Ca^{2+} influx is not stimulated by depolarisation by gramicidin D or K^+ ions (Andersson *et al.*, 1986) and the temporal characteristics of fMLP-stimulated Ca^{2+} influx are not those of a receptor-operated Ca^{2+} channel, but are more like second messenger-operated channels (SMOC) (Pittet *et al.*, 1989). It is impossible, however, to conclusively characterize the Ca^{2+} channels present without further neutrophil patch-clamping studies. Unfortunately there are however few published patch-clamping studies on human neutrophils; and without them it is difficult to demonstrate that cAMP elevation can modulate the function of specific Ca^{2+} channels.

4.3.3 Additional sites of action for rolipram and PGE_2 inhibition of human neutrophil activation

In spite of this, it is evident from the present study that rolipram and PGE_2 have a limited effect on fMLP-stimulated Mn^{2+} influx in human neutrophils, even at concentrations which almost completely inhibited Ca^{2+} influx-dependent increases in $[Ca^{2+}]_i$ and O_2^- generation. The indications are that in addition to inhibiting Ca^{2+} entry, cAMP elevation may also promote the removal of cytosolic free Ca^{2+} ions by plasma membrane and possibly endoplasmic reticular Ca^{2+} pumps. The observations from the experiments conducted in the absence of extracellular Ca^{2+} ions described in this study support this idea, such as the decline of the fMLP-stimulated Ca^{2+} transient (mobilisation of

intracellular stores) was more rapid in rolipram or PGE_2 pre-treated cells.

The neutrophil plasma membrane Ca^{2+} pump is stimulated by protein kinase C and calmodulin (Lagast *et al.*, 1984a and 1984b) but there is no evidence for its sensitivity to modulation by cAMP. Interestingly, protein kinase C increases the velocity of the pump, whereas calmodulin increases both the velocity and the Ca^{2+} ion affinity of the pump. However, there are at least 4 genes encoding plasma membrane Ca^{2+} pumps (PMCA1-4) with different tissue gene-splice variants of the most abundant type PMCA1 whose function exhibits differential sensitivity to cyclic nucleotide regulation; for example, in erythrocyte ghosts PKA-dependent phosphorylation increases the velocity and the affinity of the pump for Ca^{2+} ions (James *et al.*, 1989).

The Ca^{2+}-ATPases (pumps) on endoplasmic or sarcoplasmic reticulum differ from those in the plasma membrane. The intracellular Ca^{2+}-pumps are of three types; SERCA1, SERCA2 and SERCA3 (Grover & Khan, 1992). SERCA1 and SERCA2 pumps are regulated by cAMP dependent phosphorylation of phospholamban, whilst SERCA3 is regulated by cAMP-dependent protein kinase (PKA). The dephosphorylated form of phospholamban inhibits the SERCA pump, and phosphorylation of phospholamban by PKA would therefore lessen the restraint on Ca^{2+} pump activity. It is not surprisingly therefore that the SERCA2b isoform has been detected in human neutrophils (Stendahl *et al.*, 1994). In adherent non-phagocytosing neutrophils the Ca^{2+}ATPase was evenly distributed throughout the cytosol; during the early stages of phagocytosis the distribution was concentrated adjacent to the forming phagosome; and after complete ingestion, the Ca^{2+}ATPase was focussed around the periphery of phagosomes. The regional and temporal changes in the distribution of SERCA2b pumps suggests that they may regulate local changes in $[Ca^{2+}]_i$ during neutrophil activation, and that cAMP elevation does modulate neutrophil endoplasmic reticulum Ca^{2+} pump function. Furthermore, fMLP inhibits the uptake of Ca^{2+} ions by a Ca^{2+}-ATPase (pump) in human neutrophil lysosomes which has a high affinity for Ca^{2+} (K_m=107nM) and therefore is active at

resting $[Ca^{2+}]_i$ (Klempner, 1985) and these intracellular pumps may have a physiological role in maintaining a low normal resting $[Ca^{2+}]_i$.

In addition to the inhibition of fMLP-stimulated increases in $[Ca^{2+}]_i$, cAMP elevation may also have other modulatory sites of action. With regard to O_2^- generation, there is some evidence that the assembly and function of the O_2^- generating enzyme complex, NADPH-oxidase, may itself be regulated by cAMP-elevation. Protein kinase A phosphorylation of the Rap-1A regulatory protein inhibits Rap1A binding to the cytochrome b_{558} component of NADPH oxidase (Quilliam *et al.*, 1991 and Bokoch *et al.*, 1991). Cytochrome b_{558} is the terminal carrier of the electron transfer chain of the oxidase complex, and activation of this complex requires the association of cytochrome b_{558} to $p47^{phox}$, $p67^{phox}$, an NADPH-binding protein and a GTP-binding protein. In some forms of chronic granulomatous disease where cytochrome b_{558} is absent; the neutrophils are unable to generate O_2^- anions which underlines the importance of cytochrome b_{558} in the oxidase system (Morel *et al.*, 1991).

The discrepancy in the weakness of PGE_2 as an inhibitor of fMLP-stimulated Mn^{2+} influx, compared with its potency and efficacy as an inhibitor of fMLP-stimulated increase in $[Ca^{2+}]_i$ and O_2^- generation contrasts to the consistency of the inhibitory effects of rolipram. This difference, in addition to the possible effects of Mn^{2+} ions on adenylate cyclase and PDE function may relate to differences in the mechanism and also to the spatial localisation of cAMP elevation stimulated by PGE_2 and rolipram. Most measurements of cAMP increases are of total cellular cAMP content and do not reflect regional localised increases in cAMP (Barsony & Marx, 1990) which are likely to be different as PGE_2 stimulates adenylate cyclase whilst rolipram inhibits the metabolism of cAMP by the phosphodiesterase. Any temporal differences between PGE_2 and rolipram stimulated increases in cAMP are unlikely to result in the differences in inhibitory activity on Mn^{2+} influx as they had identical effects on the fMLP-stimulated increases in $[Ca^{2+}]_i$

which occurred over the same time-course as the Mn^{2+} influx experiments. Furthermore, inhibition of Ca^{2+} influx is not the only mechanism employed by cAMP-elevating agents, and effects on other intracellular activation pathways cannot be excluded.

Thus, cAMP elevation modulates receptor-activated (i.e. fMLP) Ca^{2+} entry, although the effect is relatively small compared with the inhibition of increases in $[Ca^{2+}]_i$ and O_2^- generation. However attenuation of Ca^{2+} entry represents only one mechanism of action; whilst the body of evidence suggests that there are additional modulatory sites where cAMP can exert its inhibitory effects on neutrophil activation.

4.3.4 Comparison of thapsigargin and fMLP-stimulated superoxide generation and increase in $[Ca^{2+}]_i$ by human neutrophils

In the present study, thapsigargin-stimulated O_2^- generation by human neutrophils was completely abolished in the presence of EGTA suggesting that the response was entirely dependent on Ca^{2+} influx. In contrast, fMLP-stimulated O_2^- generation was only partially reduced by the removal of extracellular free Ca^{2+} ions. These data suggest that there are differences in the signal transduction pathways utilised by these two stimuli.

Furthermore, the addition of extracellular Ni^{2+} ions had no effect on fMLP-stimulated O_2^- generation but completely inhibited thapsigargin-stimulated O_2^- generation; indicating additional differences in the types of Ca^{2+} channels they activate in the human neutrophil. However, Ni^{2+} ions have been reported to suppress fMLP-stimulated human neutrophil chemotaxis (Merritt *et al.*, 1991) but not fMLP-stimulated neutrophil adhesion (Davies *et al.*, 1990).

Although these two stimuli activate neutrophils by different mechanisms, fMLP (via a plasma membrane receptor) and thapsigargin (inhibition of endoplasmic Ca^{2+}-ATPase); they are reported to induce identical kinetics for depletion of intracellular Ca^{2+} stores and Ca^{2+} (and Mn^{2+}) influx in human neutrophils (Demaurex, 1992). In which case, the

characteristics of their functional responses may be expected to be similar. However, with regard to O_2^- generation, this is clearly not so.

The kinetics of thapsigargin and fMLP-stimulated Ca^{2+} movements may be similar; but their profiles are different which may account for their functional differences. fMLP, as a receptor-operated stimulus elicits a rapid depletion of endoplasmic reticulum Ca^{2+} stores, and the signal initiated by fMLP interacting with its receptor is transient, and the emptying of the Ca^{2+} stores are corresponding transient. The stores emptied on fMLP stimulation are subsequently repleted by the Ca^{2+}-ATPase present on the endoplasmic reticulum.

Thapsigargin, in contrast, exerts its effects by inhibiting this same Ca^{2+}-ATPase preventing the physiological refilling of the Ca^{2+} stores eventually completely depleting the stores. Ca^{2+} influx in the human neutrophil is closely associated with the filling state of the intracellular stores of Ca^{2+} ions (Demaurex *et al.*, 1992). Thus, thapsigargin, by blocking repletion of the intracellular stores, signals the opening of Ca^{2+} channels in an effort to increase $[Ca^{2+}]_i$ and as the stores are never repleted, the signal is not terminated, the channels remain open and the Ca^{2+} influx does not cease.

Thus, the differences in sensitivity of thapsigargin and fMLP-stimulated O_2^- generation may reflect the differences in the time course of their Ca^{2+} responses and also that Ca^{2+} permeable channels with different sensitivities to Ni^{2+} permeability may be activated by the two stimuli. In which case, the effect of Ni^{2+} ions on human neutrophil chemotaxis is more difficult to interpret in light of the lack of effect on O_2^- generation. Although experimental differences cannot be excluded, such as different time courses for these two functions (5 min for superoxide and 2 hours for chemotaxis). The blockade of Ca^{2+} influx for a long period of time, such as 2 hours may cause depletion of the intracellular stores. Intracellular Ca^{2+} stores will have been discharged by fMLP during the first few minutes of stimulation; and Ni^{2+} ions will prevent the uptake of Ca^{2+} ions required to replete the stores and maintain a sufficiently raised $[Ca^{2+}]_i$ level to maintain chemotaxis

over this time period.

Finally, fMLP via activation of its membrane receptor activates signal transduction mechanisms some of which lead to elevation of $[Ca^{2+}]_i$ or are Ca^{2+}-dependent whilst other pathways are Ca^{2+} insensitive such as protein kinase C activation. Therefore, fMLP can activate human neutrophils in the absence of increases in $[Ca^{2+}]_i$, whilst thapsigargin-stimulation of neutrophils is completely dependent on increased $[Ca^{2+}]_i$. Taken in consideration with thapsigargin being a non-physiological stimulus (no termination of the activation signal); results obtained using this substance as a cell activator should be treated with care as they may not reflect normal cell responses, for instance thapsigargin stimulated increases in $[Ca^{2+}]_i$ are not sensitive to cAMP-elevating agents. However, thapsigargin-stimulated O_2^- generation was inhibited by high concentrations of rolipram and PGE_2; which suggests that cAMP-elevating agents can inhibit neutrophil activation downstream of increases in $[Ca^{2+}]_i$.

CHAPTER 5

PGE_2 AS A MODULATOR OF HUMAN AND RABBIT NEUTROPHIL ACTIVATION, AND THE DISTRIBUTION OF THE 'EP_n' RECEPTOR IN HUMAN MONOCYTES AND THE HUMAN PROMYELOCYTIC CELL LINE (HL-60)

5.1. INTRODUCTION

5.1.1 PGE_2-mediated modulation of human neutrophil activation

Neutrophils respond to stimuli with a range of responses, for instance; respiratory burst (O_2^-, Gryglewski *et al.*, 1987), degranulation (Ham *et al.*, 1983 and Hecker *et al.*, 1990), 5-lipoxygenase activation (Ham *et al.*, 1983 and Hecker *et al.*, 1990), chemotaxis (Harvath *et al.*, 1991) and adhesion (Chopra & Webster, 1988). The modulatory effects of E-type prostaglandins are not restricted to inhibition of fMLP as a stimulus nor to O_2^- generation as a function. Prostaglandins of the E-series attenuate neutrophil responses to many soluble (fMLP and PAF) and particulate (opsonised zymosan) stimuli, (Hecker *et al.*, 1990 and Gryglewski *et al.*, 1987 respectively).

The release of azurophilic granule contents (degranulation), such as β-glucuronidase, lysozyme and N-acetylglucosamine, from human neutrophils is inhibited by cAMP elevating agents, such as β-adrenergic agents, E-type prostaglandins, PDEIs and the cell permeable cAMP analogue dibutyryl cAMP (Ignarro *et al.*, 1974, Ham *et al.*, 1983 and Lad *et al.*, 1985). The activation of 5-lipoxygenase (5-LO) and subsequent generation of LTB_4 and 5-HETEs in neutrophils however appears to more sensitive than degranulation to inhibition by PGE_2 (Ham *et al.*, 1983 and Hecker *et al.*, 1990).

Thus, PGE_2 and other cAMP elevating agents may show selectivity in their ability to modulate different neutrophil functions; and may also differentially attenuate the responses to various stimuli. In this study, the potency and efficacy of PGE_2 as an inhibitor of fMLP- and opsonised zymosan-stimulated O_2^- generation by human neutrophils has been compared with PGE_2-mediated inhibition of fMLP-stimulated β-glucuronidase release and IL-8-stimulated shape change.

5.1.2 Rabbit neutrophil prostanoid receptors

The rabbit is one species in which prostaglandins have been demonstrated to have both pro-inflammatory and anti-inflammatory effects *in vivo* (Rampart and Williams, 1986). PGE_2 when co-injected with directly-acting permeability-inducing factors such as histamine and bradykinin, acts as a vasodilator potentiating plasma protein leakage into the injection sites. The enhancement of oedema by the vasodilator activity of PGE_2 is also observed with agents which stimulate neutrophil-dependent vascular permeability, such as LTB_4 and fMLP (Wedmore & Williams, 1981). This neutrophil-dependent increase in vascular permeability is abolished by rendering the rabbits neutropaenic with nitrogen mustard or anti-neutrophil serum (Wedmore & Williams, 1981).

Systemically administered prostaglandins have a selective inhibitory effect on neutrophil-dependent skin oedema in the rabbit (Rampart & Williams, 1986). These experiments were performed using infusions of PGI_2 (prostacyclin) and subcutaneously administered 15-methyl PGE_1, a stable analogue of PGE_1. However, although these findings suggest that rabbit neutrophils may express inhibitory prostanoid IP and/or EP receptors, PGE_1 is not a selective EP receptor agonist and its effects are not easily attributed to either receptor. In addition, agonist activity for PGE_1 has been described at the IP receptor on guinea-pig platelets (Eglen & Whiting, 1988). Thus, from these observations, it is unclear which prostanoid receptors are present on rabbit neutrophils. The characterization of the prostanoid receptors present on rabbit neutrophils, therefore, is an important issue to address.

In vitro studies (Fantone *et al.*, 1984) demonstrated that 15-methyl PGE_1 inhibited rabbit neutrophil (elicited from the peritoneal cavity) degranulation and O_2^- stimulated by fMLP, OZ and PMA. In addition, PGE_2 and PGI_2 also inhibited degranulation and to a similar extent suggesting that rabbit neutrophils may express inhibitory prostanoid EP and IP receptors. If the rabbit neutrophil EP receptor is similar to that on the human neutrophil, the rabbit may prove to be a suitable species for the demonstration of anti-inflammatory

activity of selective agonists for this receptor. Furthermore, the characterization of rabbit EP receptors in general, would generate information on the species distribution of the 'EP_n' receptor and therefore provide evidence that the human neutrophil EP receptor was either a human homologue of the classical EP_2 receptor, originally characterized in the cat trachea (Coleman *et al.*, 1987) or was a novel EP receptor subtype.

5.1.3 PGE_2 receptors on mature human peripheral blood monocytes

The immunomodulatory effect of PGE_2 on leukocyte function is not confined to the neutrophil, but extends to the monocyte (Gordon *et al.*, 1976, Endres *et al.*, 1991) and lymphocyte (Gordon *et al.*, 1979, van Tits *et al.*, 1991). These mononuclear cells are more long-lived than neutrophils; and have more orchestrative roles in the inflammatory response even though monocytes and neutrophils share the same progeny. Mononuclear cells are generally considered more important in chronic inflammatory disorders than polymorphonuclear leukocytes such as neutrophils, and so modulation of monocyte and lymphocyte activation may have greater therapeutic potential.

The role of PGE_2 on monocyte function is more complex than with neutrophil activation. PGE_2 not only regulates monocyte responses to inflammatory stimuli (Gordon *et al.*, 1976, Scales *et al.*, 1989); but is also a major product of monocyte activation (Gordon *et al.*, 1976). In contrast, most human neutrophil-derived eicosanoids are generated via the lipoxygenase pathway; whilst cyclo-oxygenase products are minor products (Stenson & Lobos, 1982) compared to 5-lipoxygenase metabolites. The ability of exogenous PGE_2 to selectively inhibit LPS-stimulated TNF_α but not IL-1 generation by human monocytes (Endres *et al.*, 1991) suggests that monocyte function pathways may be differentially regulated. Type IV PDEIs exhibit a similar profile of activity to PGE_2, such as inhibition of LPS-stimulated TNF_α generation and inhibition of fMLP-stimulated O_2^- generation (Elliott & Leonard, 1989). Thus, it is possible that PGE_2 may also elevate cAMP levels in human monocytes and therefore share a common mechanism of action with PDEIs; as

appears to be the case with human neutrophils (chapter 4).

Monocyte responses such as TNF_{α} generation, generally follow a longer time-course than neutrophils; hence the effect of inhibitors is not always as easily interpreted as their chemical stability may be critical in their activity. Thus, the human monocyte prostanoid EP receptor may be more easily characterized by determining the more rapid effects of the agonists and antagonists on cAMP accumulation. Characterization of the monocyte prostanoid EP receptor would also provide more knowledge about the distribution of the 'EP_n' receptor amongst human leukocytes, and the more general anti-inflammatory and immunomodulatory potential of agonists at this receptor.

5.1.4 Characterization of PGE_2 receptors present on undifferentiated HL-60 cells

The human promyelocytic leukaemic cell line, HL-60, represents a common progenitor cell which monocytes and neutrophils share. There are other monocyte-like leukocytic cell lines, for instance, U937, THP-1 and mono mac-6, but these are committed cell lines which can only be matured into monocyte-like cells, which suggests that the HL-60 cell represents a more immature promyelocyte. In their undifferentiated form, HL-60 cells are unable to mount a functional response to inflammatory stimuli as a consequence of there being few receptors for the stimuli and/or poor coupling of the receptors to effector mechanisms (Collins *et al.*, 1979, Newburger *et al.*, 1984). HL-60 cells can be differentiated into neutrophil-like or monocyte-like cells by using different maturation conditions (Collins *et al.*, 1979) and are then functionally competent to respond to stimuli.

HL-60 cells have been used in numerous studies as a source of neutrophilic and monocytic cells for biochemical and pharmacological studies. Their expression of prostanoid receptors was investigated in this study as a potentially useful cell line for the characterization of prostanoid receptors and screening of novel prostanoid agonists. The advantage of the HL-60 cell line would be the lack of inherent variability associated with neutrophils (or monocytes) from donors caused by natural variability of the receptor

density, and possibly also in the multiple types of prostanoid receptors expressed in human neutrophils (chapter 3). Thus, human leukocytes possess a multitude of prostanoid receptors such as DP coexisting with 'EP_2'/'EP_n' receptors in neutrophils and DP, IP coexisting with 'EP_2', 'EP_n' or 'EP_4' receptors in monocytes respectively. The relative numbers of each receptor type expressed differ between individuals, which obviously causes problems in receptor characterization using these cells.

It is evident that even in their undifferentiated form, HL-60 cells respond to PGE_2 and other prostanoid EP agonists with an elevation in cAMP levels. Hollingsworth & De Vries, (1992), suggested that the receptor responsible was the EP_2 subtype. However, the characterization was based on the potency order of misoprostol and 11-deoxy PGE_1 compared to PGE_2, and the inactivity of sulprostone. The EP receptor characterization in the HL-60 is therefore incomplete as selective EP_2 agonists and prostanoid receptor antagonists were not used and warrants more thorough classification.

5.1.5 Aims

The aims of this chapter were to:-

1. Profile the inhibitory activity of PGE_2 on human neutrophil activation by comparing its potency and efficacy as an inhibitor of O_2^- generation stimulated by soluble and particulate stimuli, and as an inhibitor of neutrophil degranulation and shape change.

2. Characterize the prostanoid EP receptor on rabbit neutrophils to determine the species diversity of the EP_n receptor originally characterized on the human neutrophil (in chapter 3).

3. Characterize the prostanoid EP receptor present on human peripheral blood monocytes and undifferentiated HL-60 cells to determine the distribution of the EP_n receptor on human leukocytes.

5.2 RESULTS

5.2.1 Effect of PGE_2 on human neutrophil activation

5.2.1.1 Effect of PGE_2 on superoxide generation by human neutrophils stimulated by soluble and particulate stimuli

C5a and fMLP have a similar potency as stimuli of O_2^- generation by human neutrophils (Fig 5.1), either agent (10^{-7}M) stimulated approximately 90% of the maximum response (n=2 and n=3 respectively). However, in some donors fMLP was the more efficacious stimulus, fMLP and C5a (10^{-7}M) stimulated the generation of 26.4±2.1 and 12.0±0.1 nmol O_2^- 10^6 neutrophils^{-1} respectively (n=3, $p<0.05$). PGE_2 was an equieffective inhibitor of C5a and fMLP-stimulated O_2^- generation by human neutrophils (Fig 5.2). The comparative $p[A_{50}]$ values for PGE_2 against C5a and fMLP were 7.7±0.3 and 7.1±0.1 respectively ($p>0.05$, n=3), and the maximum inhibitions observed were similar, 85.0±7.9% and 93.3±1.5% respectively ($p>0.05$, n=3).

Opsonised zymosan concentration-dependently stimulated superoxide generation by human neutrophils (Fig 5.4). A concentration of 1mg ml^{-1} opsonised zymosan (15 min stimulation period) was chosen to investigate the effect of inhibitors which stimulated 23.7±2.6 nmol O_2^- 10^6 neutrophils^{-1}, similar to that stimulated by fMLP (see above). PGE_2 was a poor inhibitor of opsonised zymosan (OZ)-stimulated (1mg ml^{-1}) O_2^- generation by human neutrophils (21.0±1.8% inhibition at $3x10^{-5}$M, n=4), (Fig 5.3). None of the other prostanoid agonists evaluated against fMLP, misoprostol, AH13205, 11-deoxy PGE_1 or sulprostone, were able to inhibit OZ-stimulated O_2^- generation (Table 5.1). The selective type IV PDEI, RO 20-1724 also proved to be a poor inhibitor of OZ-stimulated O_2^- generation, the maximum inhibition observed was 24.3±10.9% at 10^{-5}M (n=4, Fig 5.3). Combination of PGE_2 and RO 20-1724 pretreatment of neutrophils resulted in an additive inhibition of OZ-stimulated O_2^- generation, maximum inhibition

observed was 53.0±6.0% (Fig 5.3). These data suggest that OZ-stimulated O_2^- generation by human neutrophils, in contrast to soluble stimuli, is relatively insensitive to inhibition by cAMP-elevating agents.

PAF ($2x10^{-7}M$) primed fMLP ($2x10^{-8}M$)-stimulated O_2^- generation by human neutrophils, but was unable to prime opsonised zymosan-stimulated O_2^- generation even at low levels of stimulation (Fig 5.4). PGE_2 was an effective and potent inhibitor of fMLP-stimulated O_2^- generation in PAF-primed neutrophils, with a $p[A_{50}]$ of 7.5±0.1 and maximum of 86.3±3.3% inhibition at $3x10^{-5}M$, (n=3, Fig 5.5). The potency and efficacy of PGE_2-inhibition of PAF-primed fMLP ($2x10^{-8}M$) O_2^- generation (22.8±6.4 nmol O_2^- 10^6 neutrophils^{-1}) was comparable to inhibition of fMLP ($10^{-7}M$)-stimulation in the presence of cytochalasin B (5μg ml^{-1}) (Fig 5.5).

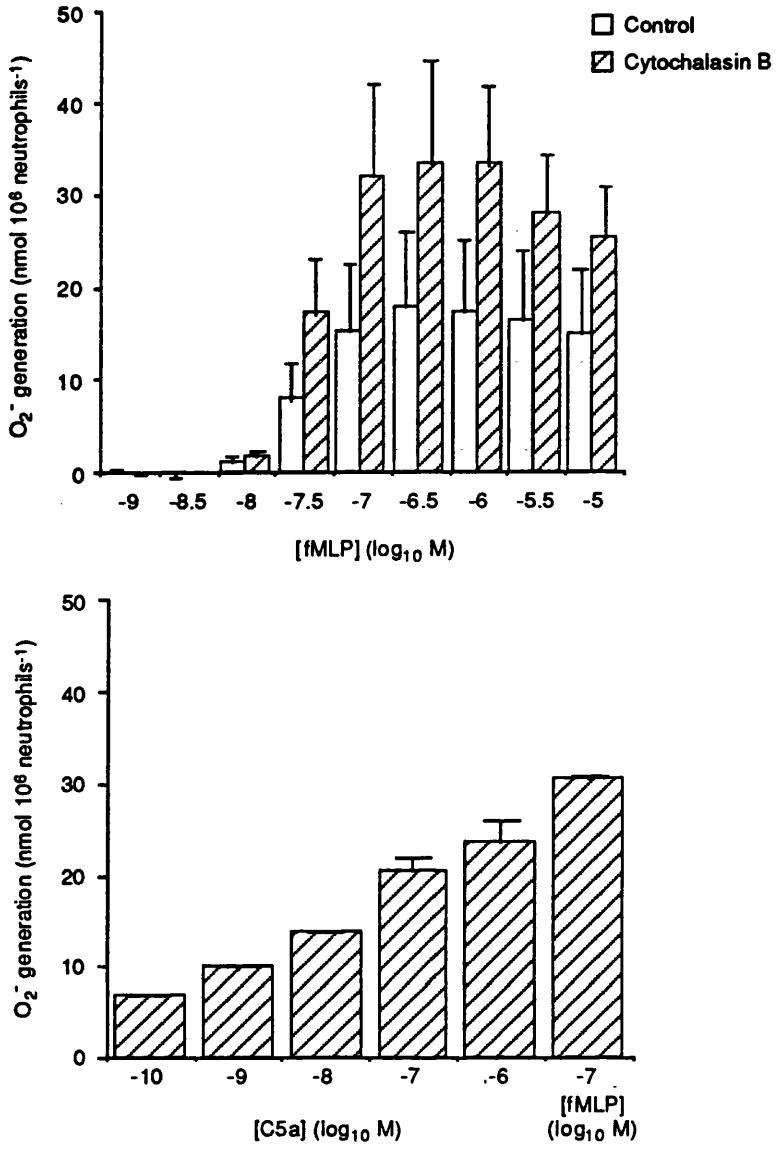


Fig 5.1 fMLP (upper panel) and C5a (lower panel)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with or without cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP (5 min, 37°C), or with cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with C5a (5 min, 37°C). The lower panel also includes fMLP (10^{-7}M) in the same experiments as C5a for comparison. Results shown are mean±s.e.m (fMLP) and mean±range (C5a) superoxide generation (nmol O_2^- 10^6 neutrophils^{-1}) of 3 and 2 separate experiments respectively performed in duplicate.

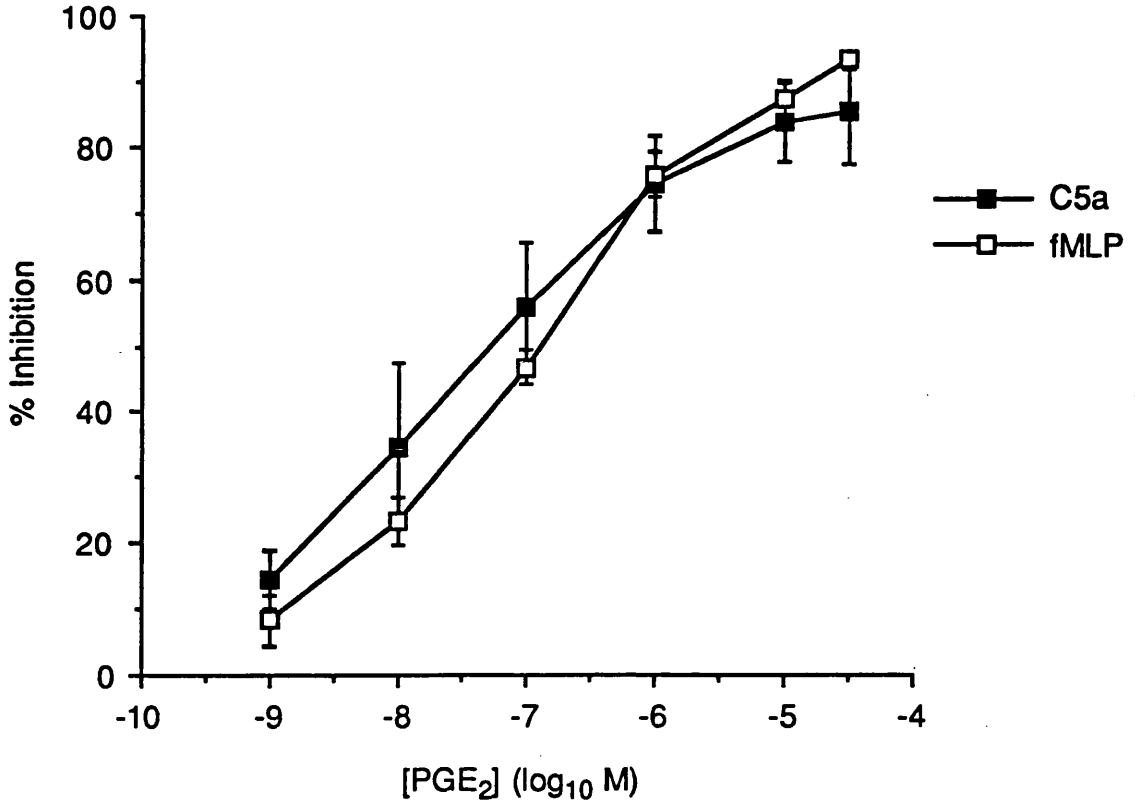


Fig 5.2 Concentration-effect curves for PGE_2-mediated inhibition of C5a (10^{-7}M) and fMLP (10^{-7}M) stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were incubated with PGE_2 in the presence of cytochalasin B (5µg ml^{-1}) prior to stimulation of superoxide generation with C5a or fMLP (5 min, 37°C). Results shown are the mean±s.e.m % inhibition of the respective control responses of 3 separate experiments performed in duplicate.

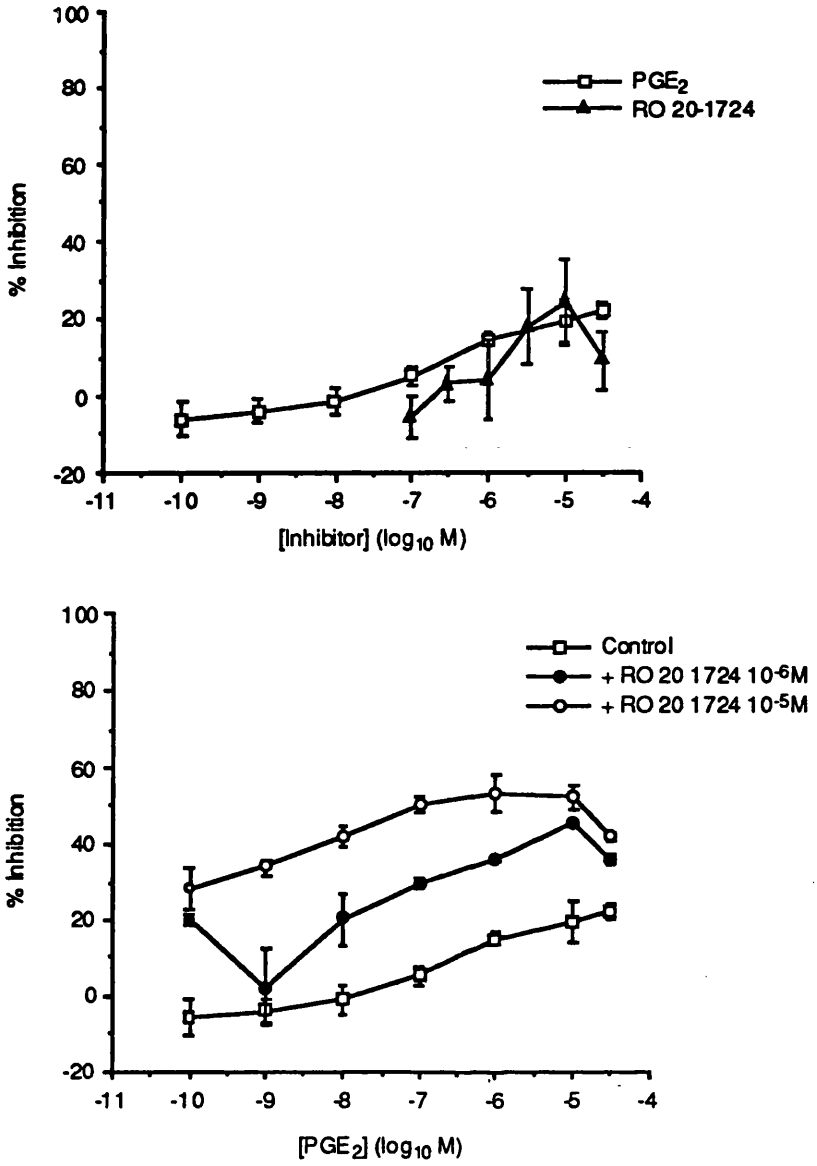


Fig 5.3 Effect of PGE_2 and the PDEI, RO 20-1724, alone (upper panel) and in combination (lower panel), on opsonised zymosan (1mg ml-1)-stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml-1) were preincubated with PGE_2 +/- RO 20-1724 for 5 min (37°C) prior to stimulation with OZ (37°C) for 15 min. Results shown are the mean±s.e.m of 4 experiments for PGE_2, 3 experiments for RO 20-1724 and 2 experiments for the combination of PGE_2 with 10^{-5}M RO 20-1724 where the results shown are the mean±range of 2 experiments, performed in duplicate.

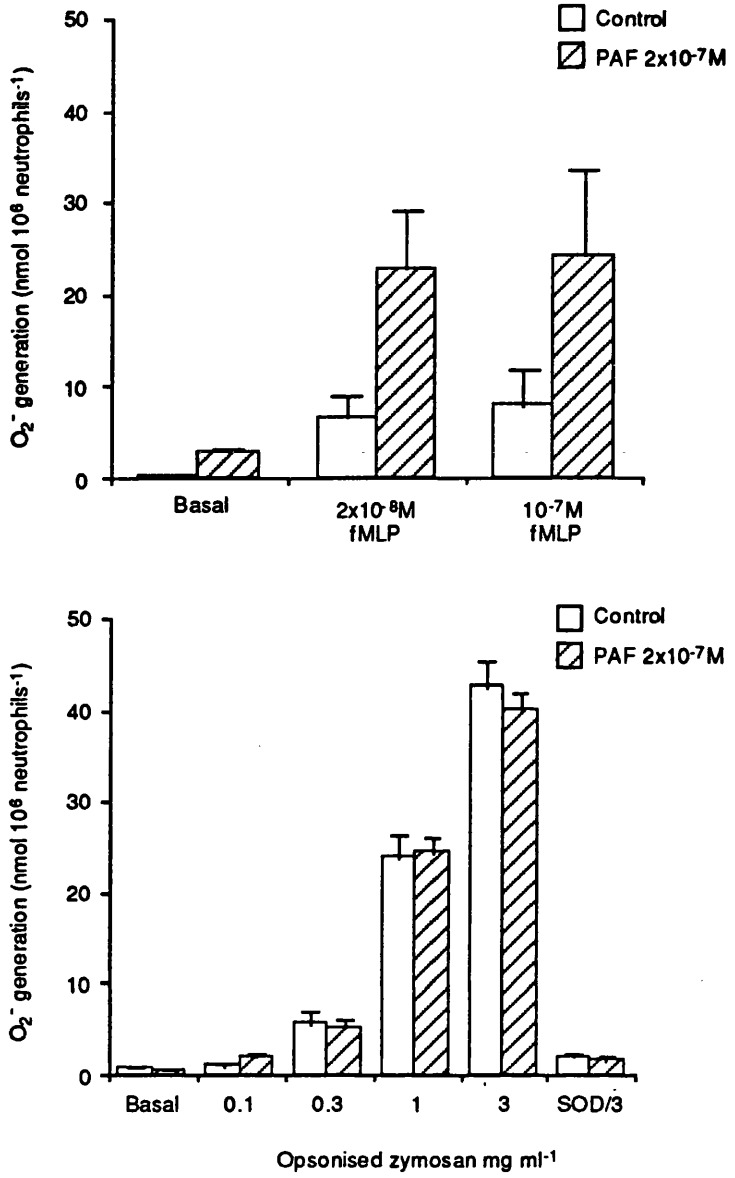


Fig 5.4 Effect of PAF (2x10-7M) priming (5 min preincubation) on fMLP (upper panel) and opsonised zymosan (lower panel) stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with PAF (5 min, 37°C) prior to stimulation with fMLP (5 min) of opsonised zymosan (0.1-3mg ml^{-1}, 15 min, 37°C) Results shown are the mean±s.e.m superoxide generation (nmol O_2^- 10^6 neutrophils^{-1}) of 4 (fMLP 2x10-8M), 3 (fMLP 10-7M) and 4 (opsonised zymosan) separate experiments performed in duplicate.

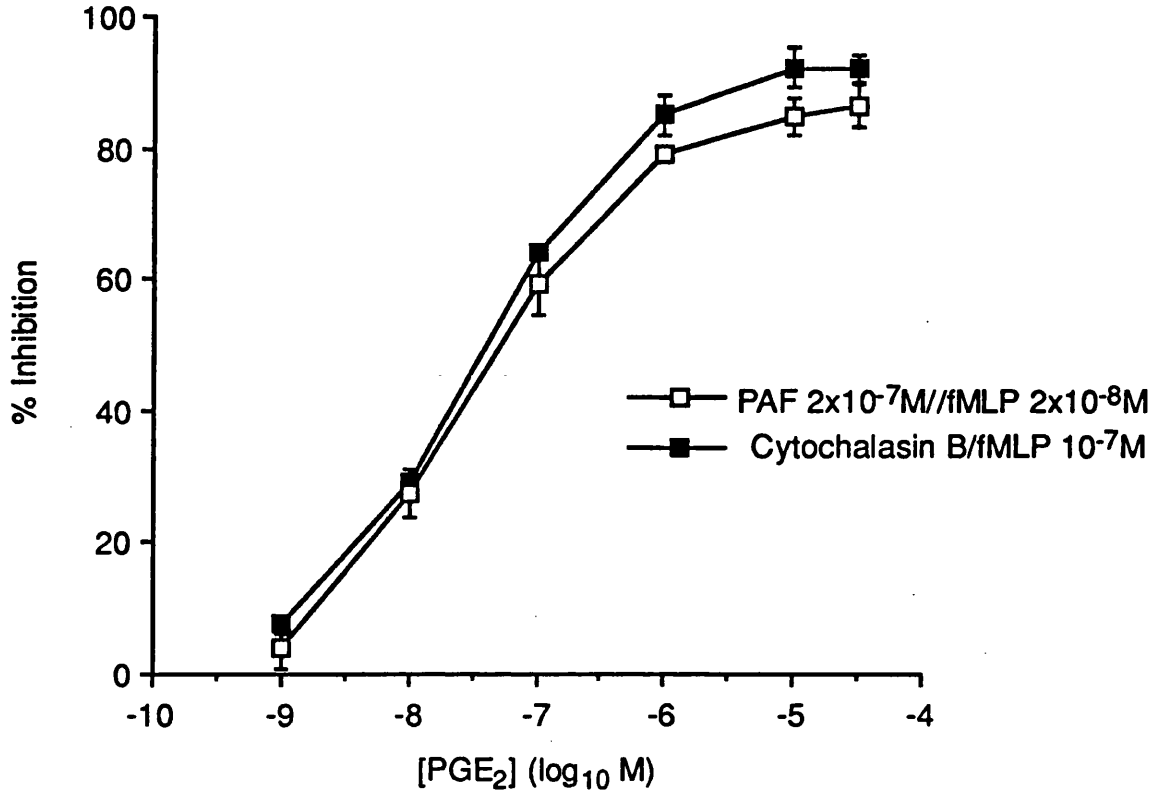


Fig 5.5 PGE_2 inhibition of fMLP ($2x10^{-8}M$)-stimulated superoxide generation by PAF ($2x10^{-7}M$)-primed human neutrophils. Neutrophils (10^6 ml^{-1}) were simultaneously incubated with PAF+/-PGE_2 (5 min, 37°C) prior to stimulation with fMLP (5 min, 37°C). Results shown are the mean±s.e.m of 3 experiments performed in duplicate, and compared to the effect of PGE_2 inhibition (5 min, 37°C) of fMLP ($10^{-7}M$)-stimulated superoxide generation (5 min, 37°C) by cytochalasin B (5μg ml^{-1}) treated neutrophils.

Log_{10} [A] M	% inhibition PGE_2	% inhibition Sulprostone	% inhibition AH13205	% inhibition Misoprostol	% inhibition 11-Dx PGE_1
-10	-7±4	-7±4	-3±2	2±7	-18±15
-9	-6±3	-29±14	-10±4	2±5	-22±18
-8	-3±3	-17±7	-8±6	-2±6	-14±17
-7	3±3	-9±12	-15±7	3±10	-9±17
-6	13±3	-25±19	-9±19	8±3	14±11
-5	15±5	-22±10	26±3	9±5	18±8
-4.5	21±2	-21±14	15±14	26±2	31±3
n	6	4	3	3	4

Table 5.1 Effect of prostanoid agonists on OZ (1mg ml^{-1}) stimulated superoxide generation by human neutrophils. Neutrophils (10^6 ml^{-1}) were preincubated with prostanoid agonists for 5 min (37°C) prior to stimulation with OZ for 15 min (37°C). Results are mean±s.e.mean % inhibition of control OZ response, n represents the number of experiments performed for each agonist in duplicate.

5.2.1.2 Effect of PGE_2 on human neutrophil degranulation

PGE_2 inhibited fMLP (10^{-7}M)-stimulated neutrophil degranulation measured as the release of β-glucuronidase. PGE_2 was equipotent ($p[A_{50}]$ =7.0±0.1, n=4) as an inhibitor of fMLP-stimulated degranulation and O_2^- generation, although the maximum inhibition of β-glucuronidase release was significantly lower ($p<0.001$), 61.0±3.7% inhibition at $3x10^{-5}$M, n=4) (Fig 5.6) compared to O_2^- generation (91.2%, n=5 cited in chapter 4).

5.2.1.3 Effect of PGE_2 on human neutrophil shape change

Neutrophil shape change (polarisation) is functionally related to chemotaxis (Wymann *et al.*, 1990) as both require changes in the cytoskeleton. fMLP (10^{-11}-10^{-8}M) and IL-8 (10^{-10}-10^{-8}M) stimulated an increase in the proportion of polarised neutrophils (Fig 5.7), quantified manually and by flow cytometry respectively.

PGE_2 (10^{-9}-10^{-4}M) inhibited human neutrophil polarisation stimulated by submaximal concentrations of IL-8 ($3x10^{-10}$M and 10^{-9}M) as quantified by flow cytometry (n=3 donors) (Table 5.2). The degree of inhibition was dependent on the strength of the stimulation, less inhibition was observed with 10^{-9}M IL-8 than with $3x10^{-10}$M IL-8. Similarly, PGE_2 (10^{-5}M) inhibited fMLP (10^{-9}M)-stimulated polarisation by 45.3±5.0% ($p<0.05$); but did not inhibit polarisation stimulated by 10^{-8}M fMLP (0.7±2.2% inhibition) (n=3).

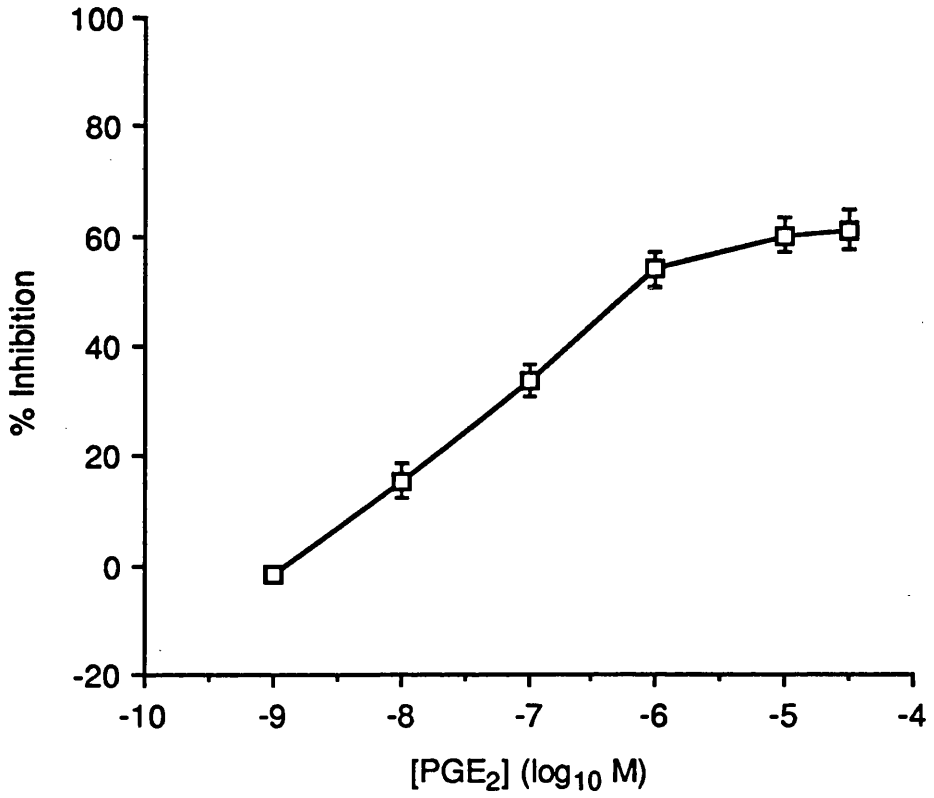


Fig 5.6 PGE_2-mediated inhibition of fMLP (10^{-7}M)-stimulated β-glucuronidase release from human neutrophils. Neutrophils (10^6 ml^{-1}) were incubated with PGE_2 and cytochalasin B for 5 min (37°C) prior to stimulation with fMLP (5 min, 37°C). Results shown are the mean±s.e.m % inhibition of fMLP-stimulated β-glucuronidase release of 4 separate experiments performed in duplicate. The basal release of β-glucuronidase was 0.5±0.3% and fMLP stimulated the release of 32.5±4.1% of total β-glucuronidase content as estimated by 0.1% triton X-100 induced release.

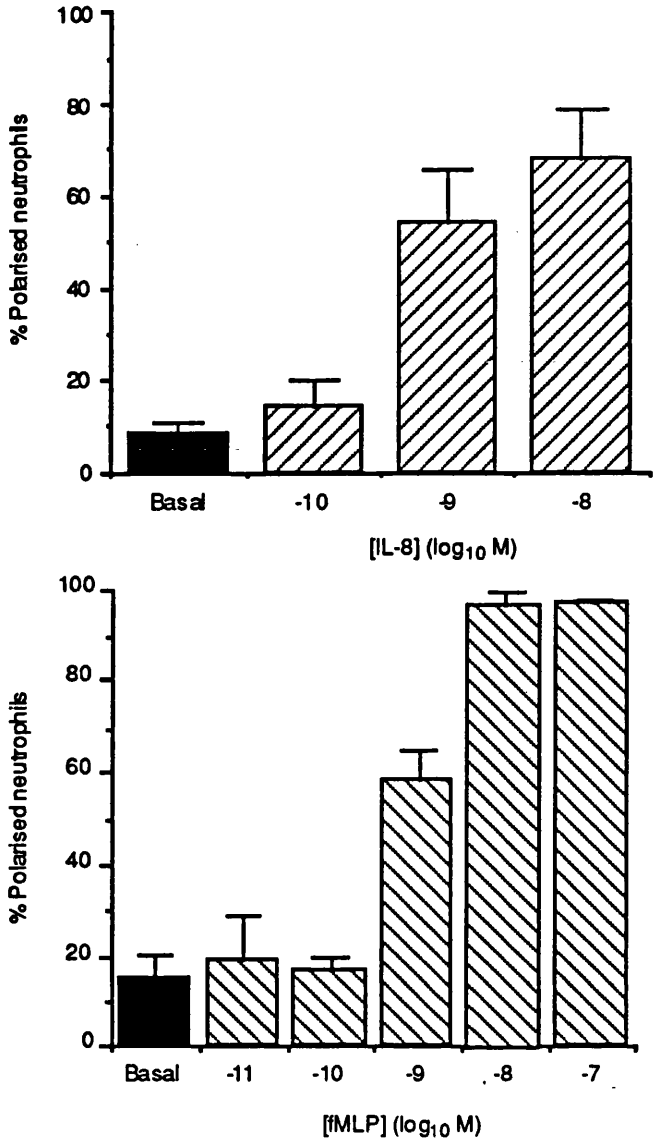


Fig 5.7 IL-8-stimulated human neutrophil shape change (polarisation) measured by flow cytometric analysis of forward light scatter (upper panel) and fMLP-stimulated human neutrophil shape change (polarisation) measured manually (lower panel). Neutrophils (10^6 ml^{-1}) were stimulated with IL-8 or fMLP (30 min, 37°C), and fixed with glutaraldehyde (1.25%) prior to analysis. Results shown are mean±s.e.m of % polarisation of 3 separate experiments performed in duplicate.

Log [PGE_2] M	% Inhibition IL-8 $3x10^{-10}M$	% Inhibition IL-8 $10^{-9}M$
-8	4±8	7±1
-7	20±15	10±4
-6	26±10	17±8
-5	40±12*	19±4
-4	53±18*	18±10

Table 5.2 PGE_2 inhibition of IL-8 stimulated human neutrophil polarisation analysed by flow cytometry (Coulter Elite Flow Cytometer). Neutrophils (10^6 ml^{-1}) were preincubated with the PGE_2 for (room temperature) prior to addition of IL-8 and incubated for 30 min (37°C). The neutrophils were fixed with glutaraldehyde (1.25%) (30 min, room temperature) and shape change (polarisation) quantified by measuring forward light scatter using flow cytometry. Results are expressed as mean±s.e.m % inhibition of the respective IL-8 control polarisation responses in 3 separate experiments performed in duplicate. Statistically significant ($P<0.05$, Student's t-test) inhibition is indicated by an asterisk.

5.2.2 Characterization of rabbit neutrophil prostanoid EP receptors

5.2.2.1 Prostanoid EP receptors on rabbit peritoneal neutrophils

The potency order for inhibition of fMLP-stimulated O_2^- generation by rabbit neutrophils elicited from the peritoneal cavity was $PGE_2 > PGI_2 > BW245C \geq PGD_2$ (Fig 5.8) and summarised in Table 5.3. None of the prostanoid receptor agonists or the adenosine receptor agonists, adenosine and NECA, were able to inhibit fMLP-stimulated O_2^- generation by more than 60% (Fig 5.8). The E/[A] curves of the prostanoid receptor agonists were not easily defined and shallow, and $p[A_{50}]$ values could not be estimated for PGD_2, PGI_2 and BW245C. The $p[A_{50}]$ for PGE_2 was 7.3±0.2 as estimated from experiments with well-defined E/[A] curves (n=5), which was similar to the $p[A_{50}]$ of 7.2±0.1 (n=30) for PGE_2 inhibition of fMLP-stimulated human neutrophil O_2^- generation (chapter 3).

5.2.2.2 Prostanoid receptors on rabbit peripheral blood neutrophils

Since elicited peritoneal neutrophils had been activated, they may have been physiologically and morphologically altered and respond differently compared to naive circulating neutrophils. Therefore the ability of PGE_2 to modulate rabbit peripheral blood neutrophil function was also determined.

fMLP-stimulated similar levels of O_2^- generation by rabbit blood-derived and peritoneal neutrophils, i.e. 10.3±1.6 and 10.3±1.1 nmol O_2^- 10^6 neutrophils^{-1} respectively. PGE_2 was a poor inhibitor of fMLP-stimulated O_2^- generation by rabbit neutrophils isolated from peripheral blood. The maximum inhibition by PGE_2 was approximately 40% at 10^{-5}M and 10^{-4}M, lower than that observed with peritoneal neutrophils (Table 5.3) and the E/[A] curve was not well defined.

Prostanoid EP receptor activation was determined using cAMP accumulation (in the presence of 5×10^{-4}M IBMX) as an alternative assay method. The basal levels of cAMP

were 239±21 fmol cAMP 10^6 neutrophils^{-1} (n=3). PGE_2 (10^{-9}-10^{-4}M) stimulated cAMP accumulation by rabbit peripheral blood neutrophils (Fig 5.9). A well-defined E/[A] curve was established with a maximum at 10^{-5}M (2,237±1,068 fmol cAMP 10^6 neutrophils^{-1} and an estimated p[A_{50}] of 6.9±0.2 (n=3), although there was a further increase in cAMP levels at 10^{-4}M PGE_2 (3,184±1,036 fmol cAMP 10^6 neutrophils^{-1}, n=3). The EP_2 selective agonists, AH13205 (p[A_{50}]=5.8±0.2, 563±355 fmol cAMP 10^6 neutrophils^{-1}, n=3) and butaprost (p[A_{50}]=5.4±0.3, 2,044±863 fmol cAMP 10^6 neutrophils^{-1}, n=3) were also able to stimulate cAMP accumulation (Fig 5.9), thus suggesting that rabbit neutrophils express 'EP_2-like' receptors positively-coupled to adenylate cyclase activation. AH 6809 ($2x10^{-5}$M) had no effect on unstimulated levels of cAMP (220±57 fmol cAMP 10^6 neutrophils^{-1}, n=3), and antagonised PGE_2-stimulated cAMP accumulation in one out of three rabbit neutrophil preparations (DR=2.0 log_{10}units, Fig 5.10). The inconsistent antagonism of PGE_2-stimulated cAMP accumulation by AH 6809 indicated that rabbit neutrophils may have a heterogenous population of prostanoid EP receptors which varies between individuals.

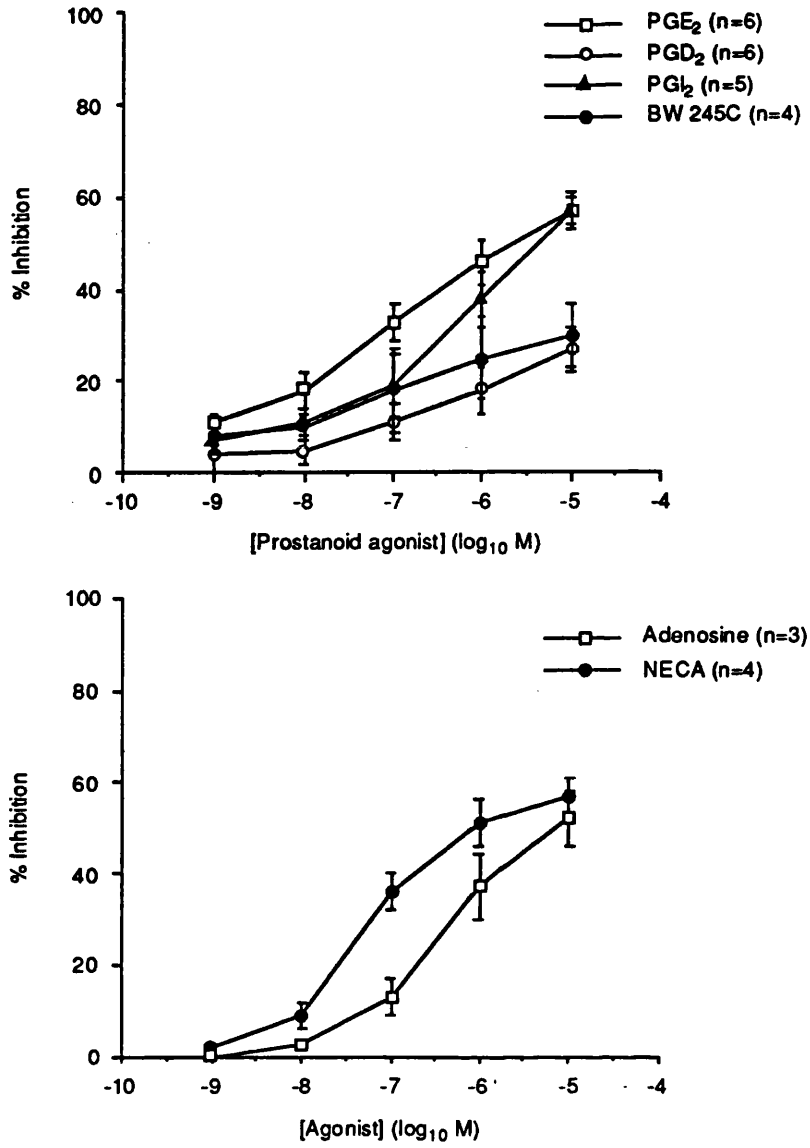


Fig 5.8 Effect of PGD_2, PGE_2, PGI_2 and the prostanoid DP receptor agonist BW 245C (upper panel), and the effect of adenosine and the adenosine receptor agonist, NECA, (lower panel) on fMLP (10^{-7}M)-stimulated superoxide generation by rabbit peritoneal neutrophils. Neutrophils ($2x10^6$ ml^{-1}) were incubated with inhibitors and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to fMLP stimulation (5 min, 37°C). Results shown are the mean±s.e.m % inhibition of the control fMLP responses of the number of experiments indicated in the legends each performed in duplicate.

Log_{10} [A] M	% Inhibition PGE_2	% Inhibition PGD_2	% Inhibition BW 245C	% Inhibition PGI_2	% Inhibition **PGE_2**
-9	11±2	4±4	8±2	7±3	**15±4**
-8	18±4	5±3	10±3	11±3	**20±5**
-7	33±4	11±4	18±9	19±7	**25±4**
-6	46±5	18±5	25±9	38±6	**32±3**
-5	57±4	27±5	30±7	57±3	**30±4**
-4	N.D.	N.D.	N.D.	N.D.	**37±4**
n	6	5	4	5	**4**

Table 5.3 Summary of the inhibitory effects of prostanoid receptor agonists and on fMLP (10^{-7}M)-stimulated superoxide generation by rabbit peritoneal neutrophils or rabbit peripheral blood neutrophils (in bold). Rabbit neutrophils ($2x10^6$ ml^{-1}) were preincubated with inhibitors and cytochalasin B (5μg ml^{-1}) for 5 min (37°C) prior to stimulation with fMLP for 5 min (37°C). Results shown are the mean±s.e.mean % inhibition of the control fMLP response, n represents the number of experiments performed in duplicate and N.D. denotes not determined. Rabbit peritoneal neutrophils and peripheral blood neutrophils generated 10.3±1.1 nmol O_2^- 10^6 neutrophils^{-1} (n=8) and 10.3±1.6 nmol O_2^- 10^6 neutrophils^{-1} (n=5) respectively.

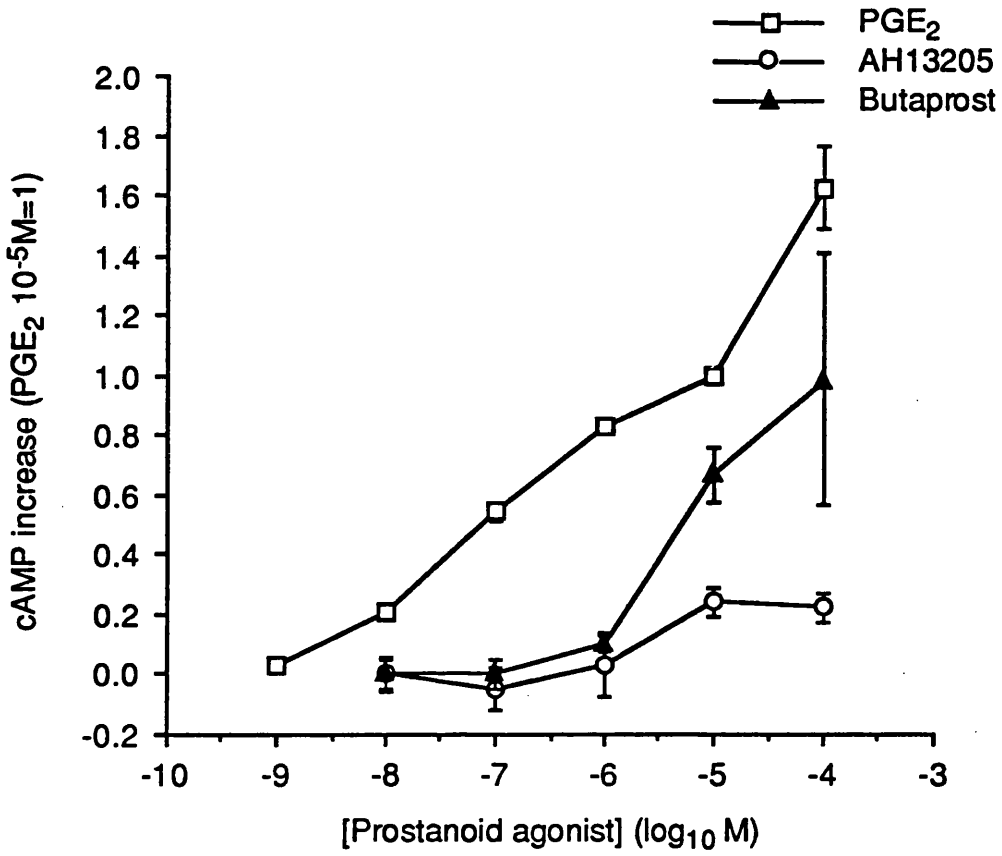
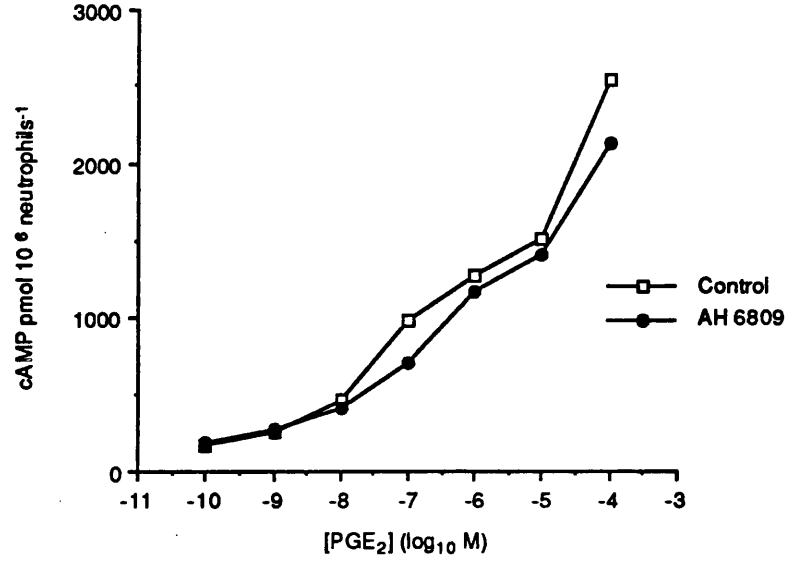
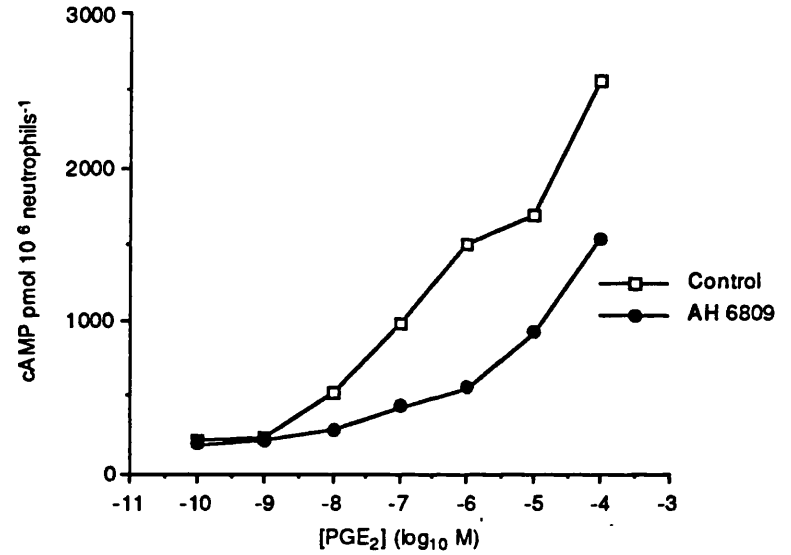
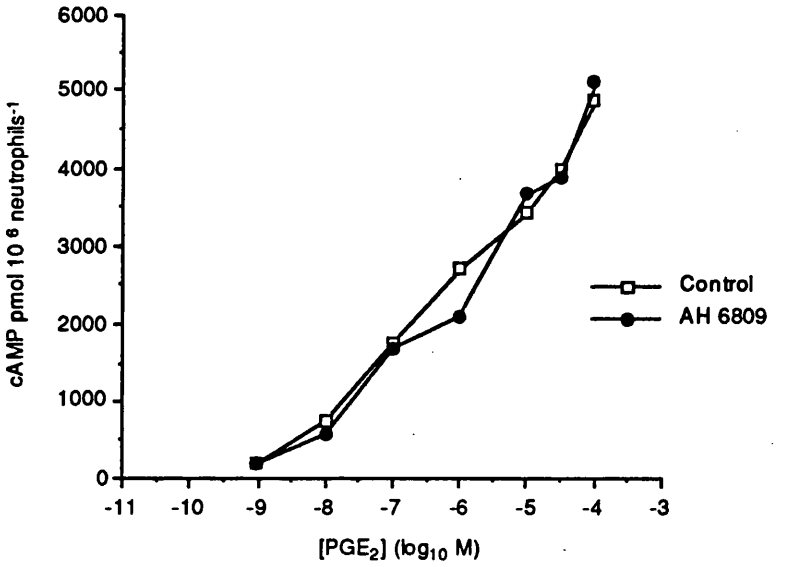


Fig 5.9 PGE_2, AH13205 and butaprost stimulated cAMP accumulation by rabbit peripheral blood neutrophils. Neutrophils ($5x10^5$ per determination) were preincubated with IBMX ($5x10^{-4}$M, 5 min, 37°C) prior to stimulation with the prostanoid EP agonists (5 min, 37°C). Results shown are mean±s.e.m cAMP increases normalised with respect to the 10^{-5}M PGE_2 response of 3 separate experiments performed in duplicate.

Fig 5.10 PGE_2-stimulated cAMP accumulation by rabbit peripheral blood neutrophils in the absence and presence of AH 6809 ($2x10^{-5}M$). Neutrophils were preincubated with AH 6809 for 30 min (room temperature) prior to PGE_2 stimulation of cAMP accumulation (5 min, 37°C) performed in the presence of IBMX ($5x10^{-4}M$). Upper middle and lower panels each show the results obtained from 3 individual rabbits, plotted as the mean of duplicate determinations (pmol cAMP 10^6 neutrophils^{-1}).


3000
2000
1000
0
cAMP pmol 10 6 neutrophils-1
-11 -10 -9 -8 -7 -6 -5 -4 -3
[PGE2] (log10 M)
Control
AH 6809


3000
2000
1000
0
cAMP pmol 10 6 neutrophils-1
-11 -10 -9 -8 -7 -6 -5 -4 -3
[PGE2] (log10 M)
Control
AH 6809


6000
5000
4000
3000
2000
1000
0
cAMP pmol 10 6 neutrophils-1
-11 -10 -9 -8 -7 -6 -5 -4 -3
[PGE2] (log10 M)
Control
AH 6809

5.2.3 Characterization of prostanoid EP receptor-mediated stimulation of cAMP accumulation in human monocytes

Donor variability was observed in the absolute amounts of cAMP generated by human monocytes both in terms of the basal levels of cAMP generation in unstimulated human monocytes of 2.9±0.6 pmol cAMP 10^6 monocytes^{-1} (n=8), and monocytes stimulated with prostanoids.

PGE_2 (10^{-9}-10^{-4}M) stimulated cAMP accumulation with a p[A_{50}] was 6.5±0.1 (n=7), calculated from the maximum cAMP increase at 10^{-5}M (50.9±3.4 pmol cAMP 10^6 monocytes^{-1}), although there was a further increase at 10^{-4}M (65.7±9.8 pmol cAMP 10^6 monocytes^{-1}) (Fig 5.11).

The selective EP_2-receptor agonists, AH13205 and butaprost, were also able to stimulate cAMP accumulation which suggests that an EP_2-(like) receptor is present on human monocytes (Fig 5.11). AH13205 appeared to be a partial agonist with an α of 0.56±0.09 (compared to the PGE_2 E_{max} at 10^{-5}M) and p[A_{50}] of 5.3±0.3 (n=4), whilst butaprost (10^{-5}M) reached a similar maximum to PGE_2, α =1.02±0.24, with a p[A_{50}] of 5.6±0.1 (n=4). The E/[A] curve however for butaprost was considerably steeper than either PGE_2 or AH13205.

To further characterize the EP receptor, the prostanoid receptor antagonists BW A868C (DP selective, 10^{-7}M), AH 6809 (DP/EP_1/EP_n, 10^{-5}M) and AH23848B (TP/EP_4, 10^{-5}M) were tested against PGE_2-stimulated cAMP accumulation. BW A868C had no effect on PGE_2-stimulated increases in cAMP, except at the highest concentration of PGE_2 tested (10^{-4}M, n=4) (Fig 5.12). The presence of DP receptors were demonstrated by PGD_2-stimulated cAMP accumulation (36.2±15.6 pmol cAMP 10^6 monocytes^{-1}) with a p[A_{50}] of 6.7±0.1 (n=4) which was completely abolished by BW A868C (Fig 5.12) suggesting

that PGE_2 ($\leq 10^{-5}M$) did not activate prostanoid DP receptors.

AH 6809 ($10^{-5}M$) produced a parallel rightward shift of PGE_2-stimulated cAMP accumulation in human monocytes with a DR=1.5±0.1 $\log_{10}$ units (n=7), and was unaffected by the presence of BW A868C ($10^{-7}M$, n=3) (Fig 5.13) suggesting that AH 6809 antagonism of PGE_2 was not mediated at the prostanoid DP receptor.

AH23848B was tested against PGE_2 at a relatively low concentration of $10^{-5}M$ compared to its EP_4 receptor pA_2 value of 4.9-5.4 (Coleman *et al.*, 1994b), as Milne *et al.* (1994) reported that $3x10^{-5}M$ AH23848B completely abolished PGE_2 stimulated increases in cAMP in human monocytes. A dose ratio of approximately 0.5 $\log_{10}$ units for AH23848B ($10^{-5}M$) against PGE_2 would have been predicted from its EP_4 receptor pA_2 value. Although the observed shift was not inconsistent with the literature pA_2 at the EP_4 receptor; it was not parallel, suggesting that AH23848Bwas non-competitive (n=7) (Fig 5.14).

To determine the selectivity of AH 6809 and AH23848B as EP-receptor antagonists, they were tested against cicaprost (selective IP receptor agonist) and compared to their effects on the PGE_2 E/[A] curve. Cicaprost was the most potent and efficacious prostanoid cAMP-stimulating agent, with a $p[A_{50}]$ of 7.8±0.2 and 221.1±53.5 pmol cAMP 10^6 monocytes^{-1} (n=8). Neither AH 6809 nor AH 23848B (both at $10^{-5}M$) had any effect on cicaprost-stimulated cAMP accumulation (n=4) shown in Fig 5.15 and summarised in Table 5.4, suggesting that both agents were selective antagonists of PGE_2-stimulation of adenylate cyclase in human monocytes.

Thus, the results suggest that human peripheral blood monocytes possess prostanoid IP receptors, lower numbers of DP receptors and at least 2 EP receptors. There appear to be 'EP_2'-like receptors as determined by the activity of AH13205 and butaprost (EP_2-selective agonists). However, as PGE_2 was antagonised by AH 6809, this 'EP_2'-like

receptor may be the same subtype as characterized in the human neutrophil (chapter 3) as EP_4 receptors are not antagonised by AH 6809 (personal communication by Simon Lydford). The presence of EP_4 receptors, as suggested by PGE_2 antagonism by AH23848B, the EP_4 receptor antagonist, albeit non-competitively, cannot be excluded.

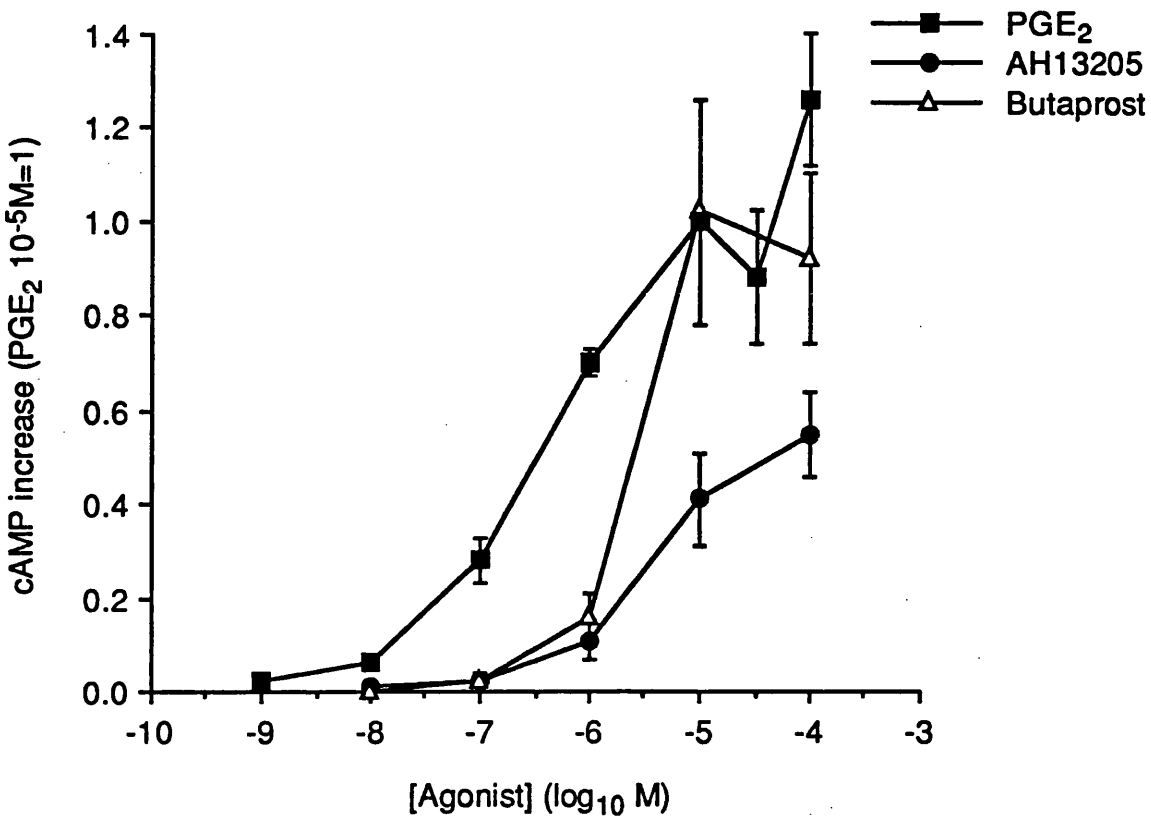


Fig 5.11 PGE_2, AH13205 and butaprost stimulated cAMP accumulation by human peripheral blood monocytes. Prostanoid agonist stimulated cAMP accumulation by human peripheral blood monocytes (10^5 per determination) was measured over a 5 min period (37°C) in the presence of IBMX ($5x10^{-4}$M). Results are expressed as a fraction of the PGE_2 cAMP response at 10^{-5}M of each respective experiment, with each point representing the mean±s.e.m of the data from 7 (PGE_2) and 4 (AH13205 and butaprost) separate experiments performed in duplicate.

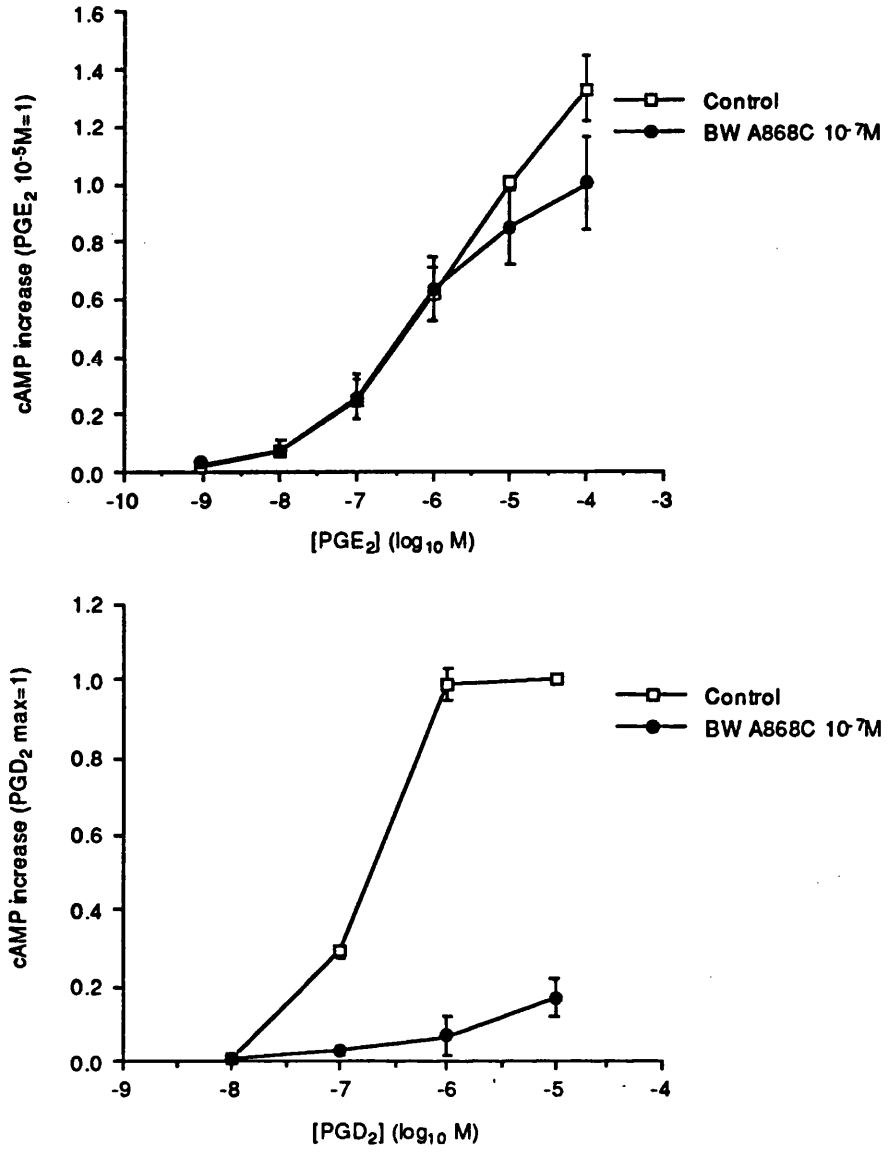


Fig 5.12 Effect of BW A868C (10^{-7}M) on PGE_2 (upper panel) and PGD_2 (lower panel) stimulated cAMP accumulation by human peripheral blood monocytes. Monocytes ($1x10^5$ per determination) were preincubated with BW A868C for 30 min (room temperature) prior to stimulation with the prostanoids for (5 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results were expressed as a fraction of the PGE_2 10^{-5}M response and the PGD_2 maximum response respectively. The data shown is the mean±s.e.m of 3 (PGE_2) and 4 (PGD_2) separate experiments performed in duplicate.

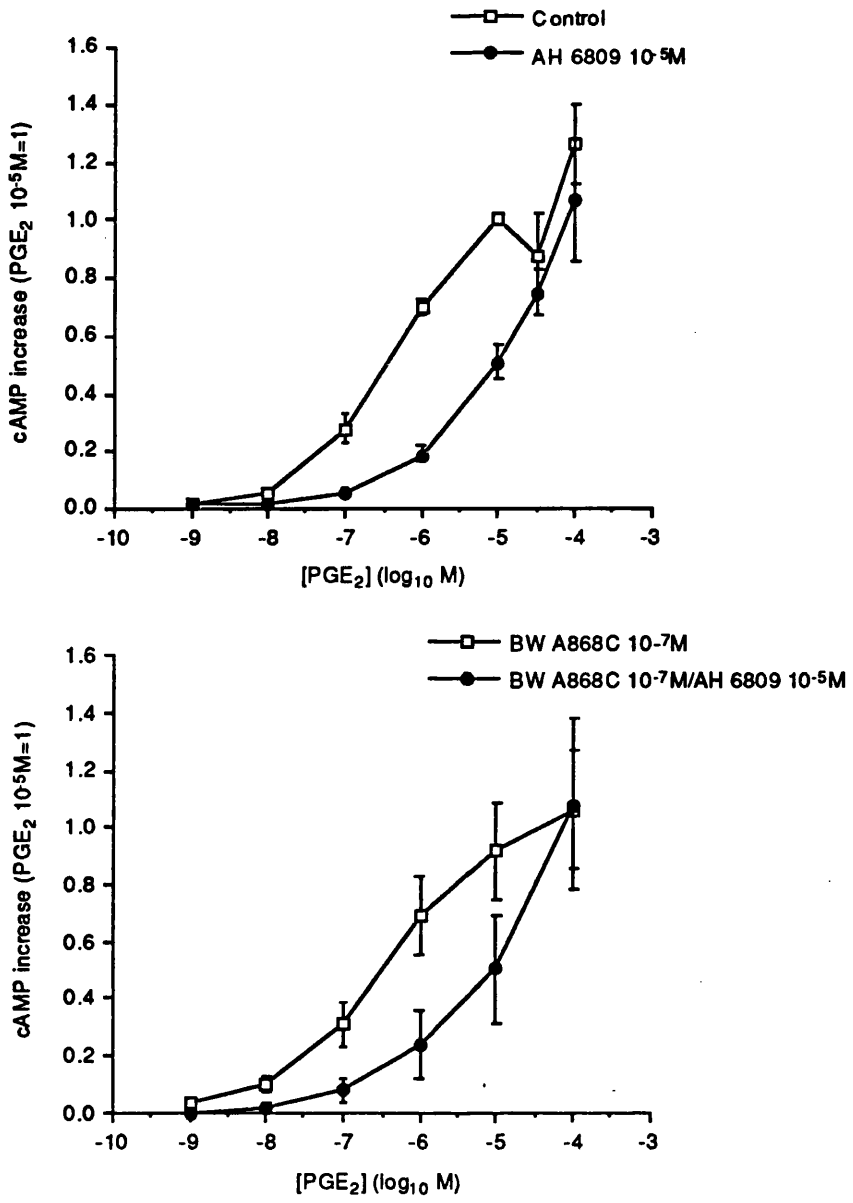


Fig 5.13 AH 6809 (10^{-5}M) antagonism of PGE_2-stimulated cAMP accumulation by human peripheral blood monocytes in the absence (upper panel) and presence of BW A868C (10^{-7}M) (lower panel). Monocytes ($1x10^5$ per determination) were preincubated with the antagonists for 30 min (room temperature) prior to stimulation with the prostanoids for (5 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results were expressed as a fraction of the control PGE_2 10^{-5}M response of each respective experiment and shown as the mean±s.e.m of 7 and 3 separate experiments respectively performed in duplicate.

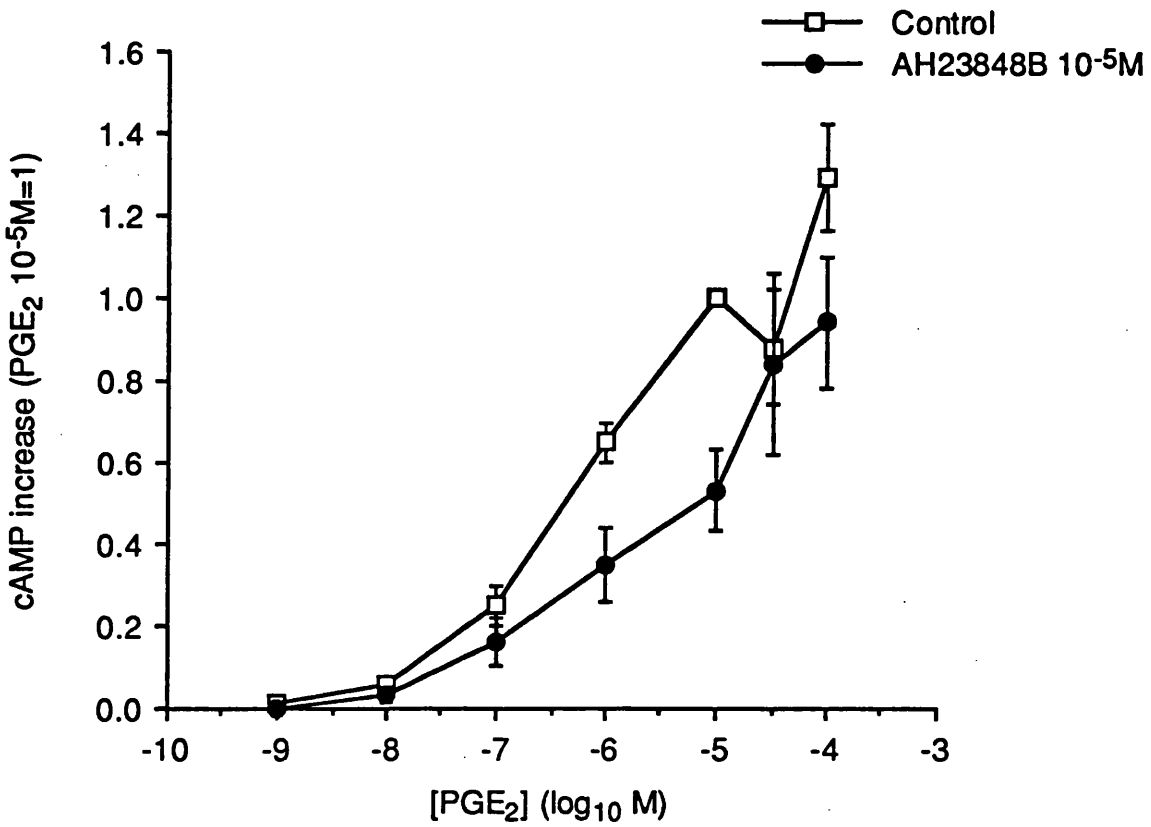


Fig 5.14 Effect of AH23848B (10^{-5}M) on PGE_2-stimulated cAMP accumulation by human peripheral blood monocytes. Monocytes ($1x10^5$ per determination) were preincubated with AH23848B for 30 min (room temperature) prior to stimulation with the prostanoids for (5 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results were expressed as a fraction of the PGE_2 10^{-5}M response and shown as the mean±s.e.m of 7 separate experiments performed in duplicate.

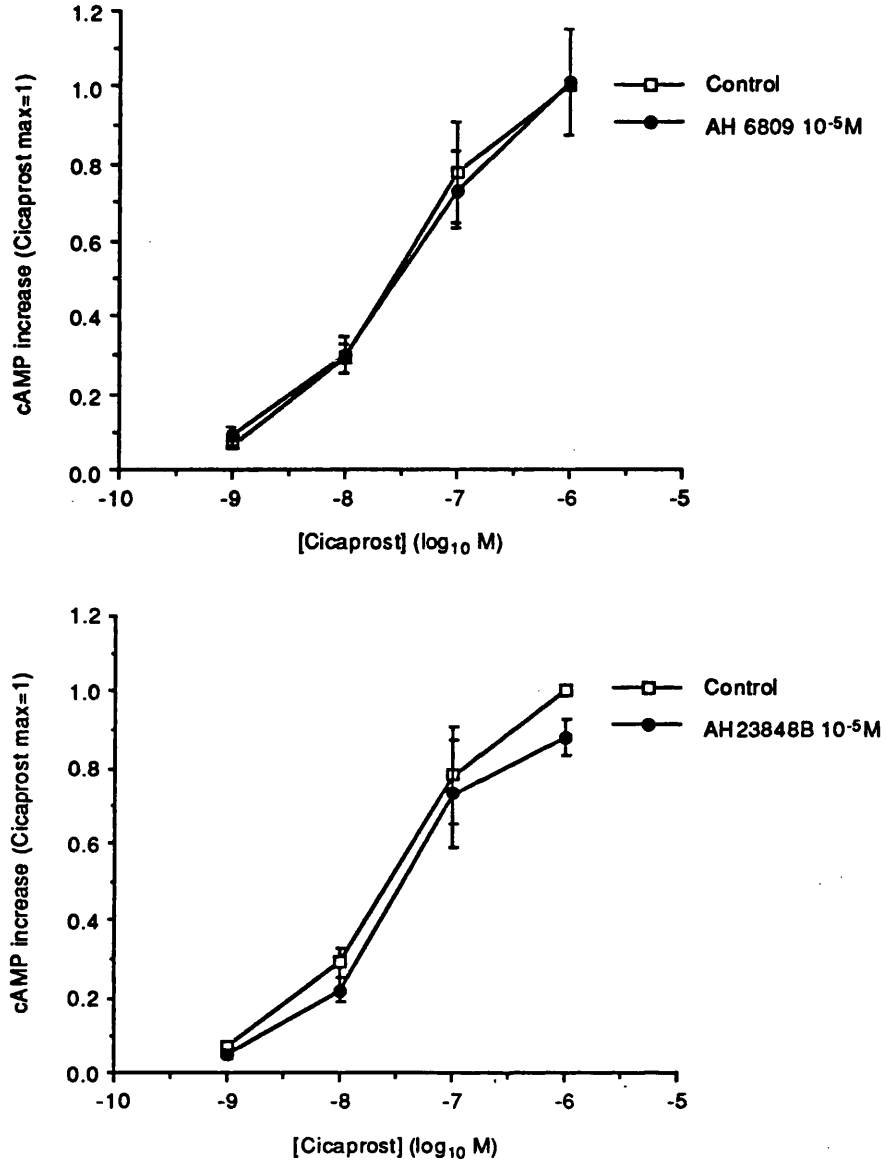


Fig 5.15 Effect of AH 6809 (10^{-5}M) (upper panel) and AH23848B (10^{-5}M) (lower panel) on cicaprost-stimulated cAMP accumulation by peripheral blood monocytes. Monocytes (10^5 per determination) were preincubated with the antagonists for 30 min (room temperature) prior to stimulation with cicaprost for (5 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results were expressed as a fraction of the cicaprost maximum cAMP response and shown as the mean±s.e.m of 4 separate experiments performed in duplicate.

Cicaprost	Cicaprost p[A_{50}]	Max increase in cAMP pmol 10^6 monocytes^{-1}
Control	7.46±0.18	286±95
AH 6809 10^{-5}M	7.54±0.04	265±80
AH23848B 10^{-5}M	7.49±0.13	248±82

Table 5.4 Cicaprost (selective IP receptor agonist)-stimulated increase in cAMP by human peripheral blood monocytes in the absence and presence of AH 6809 (10^{-5}M) or AH23848B (10^{-5}M). Monocytes were incubated with the antagonists for 30 min (room temperature) prior to stimulation of cAMP accumulation by cicaprost (5 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results shown are the mean±s.e.mean data from 4 separate experiments performed in duplicate.

5.2.4 Characterization of prostanoid EP receptor-mediated stimulation of cAMP accumulation in undifferentiated HL-60 cells

The basal cAMP levels were found to be 8.0±1.2 pmol/10^6 HL-60 cells (n=22). PGE_2 (10^{-9}-10^{-4}M) increased cAMP levels accumulated over a 10 min period by 270.7±19.2 pmol/10^6 HL-60 cells with a $p[A_{50}]$ of 6.7±0.1 (n=9) (Fig 5.16). Neither PGD_2 nor cicaprost stimulated cAMP accumulation in undifferentiated cells suggesting that prostanoid DP and IP receptors were not present or not functionally coupled to adenylate cyclase on undifferentiated HL-60 cells (data not shown). In addition to AH 6809 and BW A868C, SC 19220 (EP_1 selective, pA_2 5.8) were also tested at 10^{-4}M for antagonist activity against PGE_2 stimulated increases in cAMP.

AH 6809 (10^{-5}M) had no effect on basal cAMP levels, 8.4±1.2 pmol/10^6 HL-60 cells compared to control HL-60 cells, 7.1±0.6 pmol/10^6 HL-60 cells (n=11) and caused a parallel rightward shift of the PGE_2 curve by 1.3±0.1 $\log_{10}$ units (n=9) (Fig 5.17). The selective DP receptor antagonist BW A868C (10^{-7}M) had no effect on the PGE_2 cAMP curve, the $p[A_{50}]$ was 6.7±0.1 compared to the control PGE_2 $p[A_{50}]$ of 6.6±0.1 (n=3, Fig 5.17) suggesting that PGE_2 was not acting at DP receptors. The EP_1 receptor selective antagonist, SC 19220 (10^{-4}M), did not antagonise PGE_2 stimulated cAMP accumulation, $p[A_{50}]$=6.8 (range 6.8-6.8, n=2) compared to the control PGE_2 $p[A_{50}]$ of 6.8 (range 6.8-6.8 n=2), but increased the maximum PGE_2-stimulated cAMP to 127% and 119% of the control PGE_2 maximum (n=2) (Fig 5.17).

The specificity of AH 6809 was investigated on cAMP generation stimulated by the adenylate cyclase stimulator forskolin. Forskolin was a poor stimulus of cAMP accumulation in undifferentiated HL-60 cells compared to PGE_2 (Fig 5.18). In addition to this, forskolin-stimulated cAMP generation was not antagonised by AH 6809 (10^{-5}M) (n=2).

A range of prostanoid EP agonists were tested as stimulators of cAMP accumulation, AH13205 (EP_2 selective), 11-deoxy PGE_1, misoprostol and PGA_1. The potency order of these agonists compared to PGE_2 was similar to that described for the human neutrophil cAMP increases (chapter 3), although their α values (maximum responses compared to full agonist PGE_2=1) are not the same as those observed for cAMP responses in the neutrophil (Fig 5.19). However on applying the Operational Model to both sets of data (Leff *et al.*, 1990), similar agonist potency orders were obtained (Fig 5.19) suggesting that the agonist potency order for these agonists is consistent with the EP_2 receptor subtype. The EP_1/EP_3 selective agonist sulprostone however did not stimulate cAMP accumulation in undifferentiated HL-60 cells. The levels in the presence of $1x10^{-6}$M sulprostone were 7.5±1.4 pmol $(10^6$ HL-60$)^{-1}$ compared to basal levels 7.8±1.4 pmol $(10^6$ HL-60$)^{-1}$.

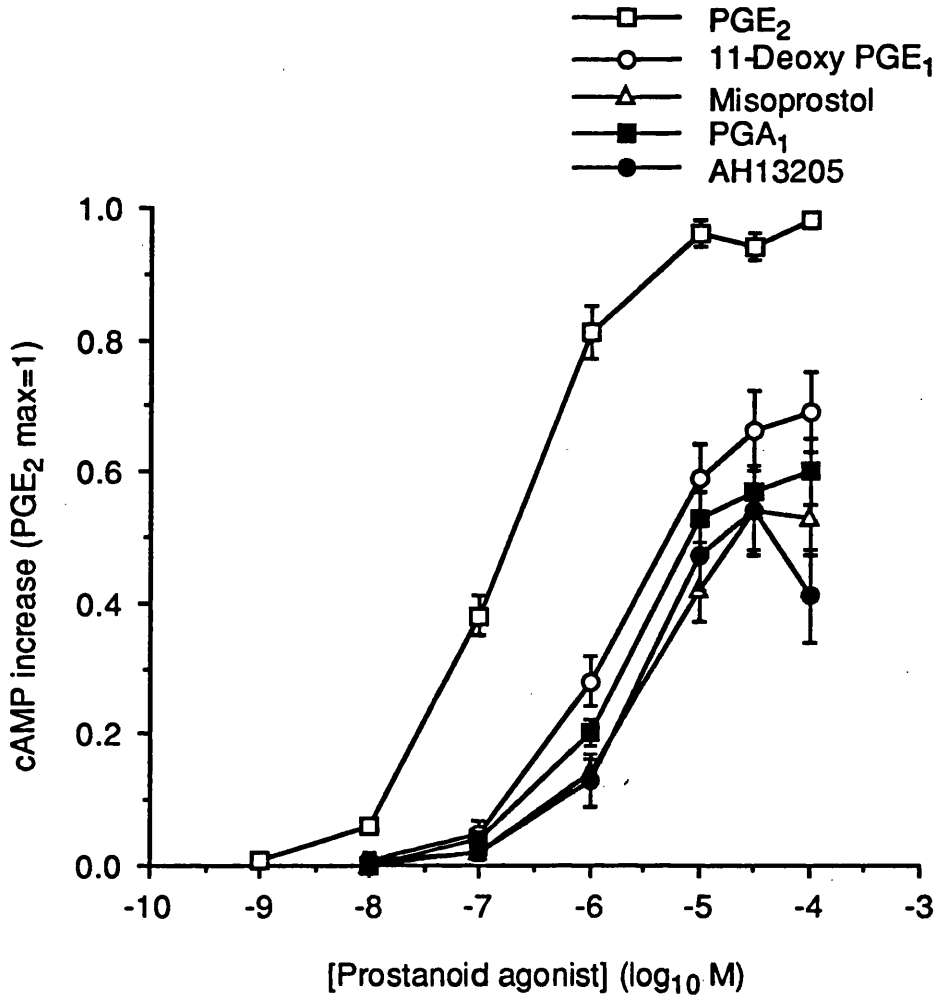
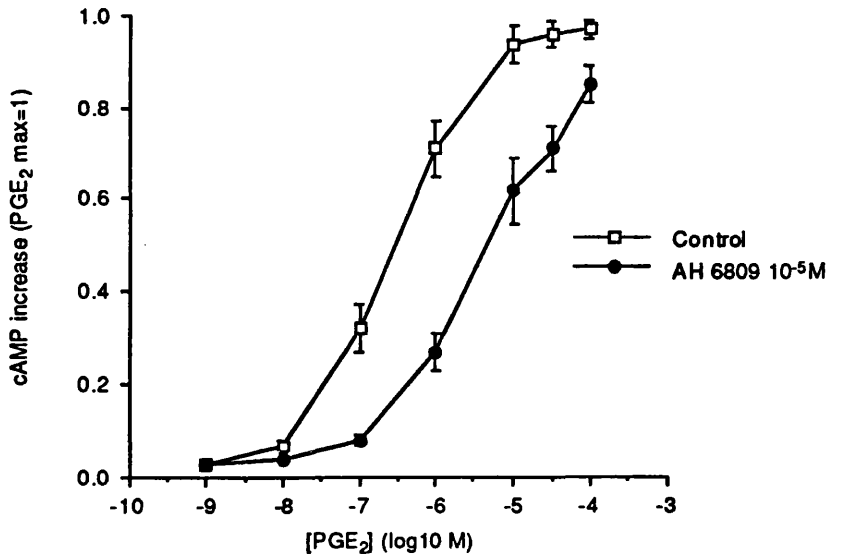
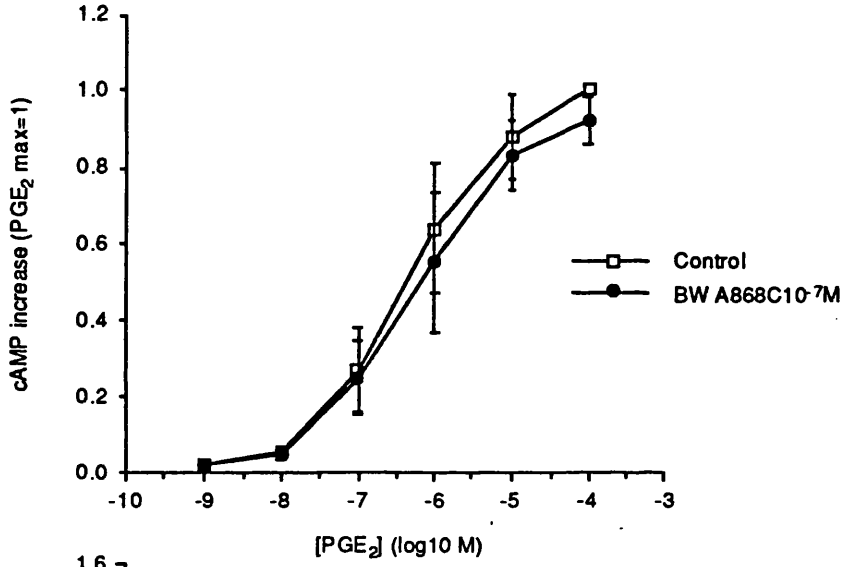
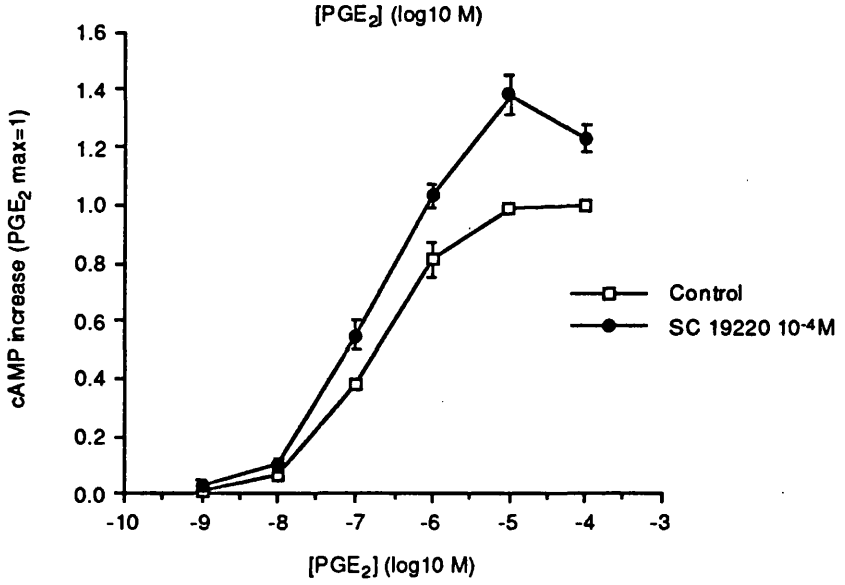


Fig 5.16 cAMP accumulation stimulated by PGE_2 and prostanoid receptor agonists 11-deoxy PGE_1, misoprostol, PGA_1 and AH13205 by undifferentiated HL-60 cells. HL-60 cells (10^6 per determination) were stimulated with the prostanoid receptor agonists for (10 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results shown represent the mean±s.e.m cAMP increase normalised with respect to the PGE_2 maximum (=1) of 5 separate experiments performed in duplicate.

Fig 5.17 Effect of AH 6809 (10^{-5}M) (upper panel), BW A868C (10^{-7}M) (middle panel) and SC 19220 (10^{-4}M) (lower panel) on PGE_2-stimulated cAMP accumulation by undifferentiated HL-60 cells. HL-60 cells (10^6 per determination) were preincubated with the antagonists for 30 min (room temperature) prior to stimulation with PGE_2 for (10 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results shown are the mean±s.e.m cAMP response normalised with respect to the control PGE_2 maximum cAMP increase of 9 (AH 6809), 3 (BW A868C) and mean±range of 2 (SC 19220) separate experiments performed in duplicate.


cAMP increase (PGE2 max=1)
1.0
0.8
0.6
0.4
0.2
0.0
-10
-9
-8
-7
-6
-5
-4
-3
[PGE2] (log10 M)
Control
AH 6809 10-5M


cAMP increase (PGE2 max=1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-10
-9
-8
-7
-6
-5
-4
-3
[PGE2] (log10 M)
Control
BW A868C10-7M


cAMP increase (PGE2 max=1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-10
-9
-8
-7
-6
-5
-4
-3
[PGE2] (log10 M)
Control
SC 19220 10-4M

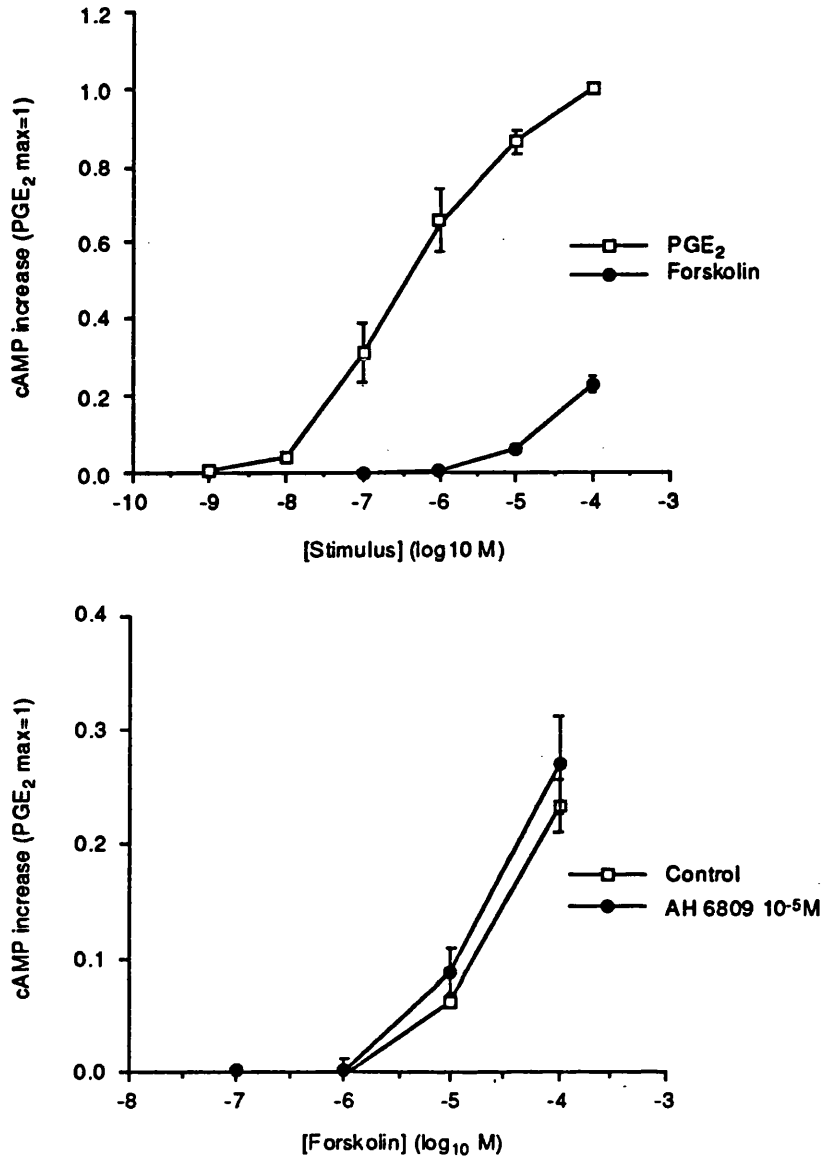


Fig 5.18 Forskolin-stimulated cAMP accumulation by undifferentiated HL-60 cells in the absence (upper panel) and presence of AH 6809 (10^{-5}M) (lower panel). HL-60 cells (10^6 per determination) were preincubated in the absence or presence of AH 6809 for 30 min (room temperature) prior to stimulation with forskolin (10 min, 37°C) in the presence of IBMX ($5x10^{-4}$M). Results shown are the mean±s.e.m cAMP increase normalised with respect to the PGE_2 maximum (as a comparison) of 3 separate experiments (upper panel) and mean±range of 2 separate experiments (lower panel) performed in duplicate.

Experimental

Neutrophil	$PGE_2 > PGA_1 > AH13205 > 11\text{-Deoxy } PGE_1 > Misoprostol$				
$p[A_{50}]$	6.8	6.3	5.8	5.6	5.2
s.e.m	±0.1	±0.2	±0.1	±0.2	±0.2
α	1.0	0.62	0.34	1.0	1.0
HL-60	$PGE_2 > 11\text{-deoxy } PGE_1 \approx PGA_1 > Misoprostol \geq AH13205$				
$p[A_{50}]$	6.8	5.7	5.7	5.5	5.5
s.e.m	±0.1	±0.1	±0.1	±0.1	±0.1
α	1.0	0.73	0.62	0.62	0.57

Simulated

Neutrophil	$PGE_2 > 11\text{-deoxy } PGE_1 \approx PGA_1 > Misoprostol \approx AH13205$				
$p[A_{50}]$	8.8	7.6	7.6	7.2	6.9
s.e.m	±0.1	±0.2	±0.1	±0.2	±0.2
α	1.0	1.0	1.0	1.0	1.0
HL-60	$PGE_2 > 11\text{-deoxy } PGE_1 \approx PGA_1 > Misoprostol \approx AH13205$				
$p[A_{50}]$	8.8	7.6	7.5	7.2	7.2
s.e.m	±0.1	±0.1	±0.1	±0.1	±0.1
α	1.0	1.0	1.0	1.0	1.0

Fig 5.19 Comparison of the potency orders of prostanoid EP agonists mediating cAMP accumulation by human neutrophils (data from chapter 3) and undifferentiated HL-60 cells before and after simulation of increased receptor reserve.

5.3 DISCUSSION

The results presented in this chapter suggest that:-

1. PGE_2 selectively inhibits the activation of human neutrophils, and is not a global modulator of neutrophil function. PGE_2 inhibited fMLP- and C5a-stimulated O_2^- generation; but was a poor inhibitor of OZ-stimulated O_2^- generation. Although PGE_2 was a potent and efficacious inhibitor of fMLP-stimulated O_2^- generation, fMLP-stimulated β-glucuronidase release was inhibited by a lower degree but with similar potency. In addition, PGE_2 was a poor inhibitor of IL-8- and fMLP-stimulated neutrophil polarisation; the degree of inhibition was inversely proportional to the concentration of IL-8 and fMLP, suggesting functional antagonism.

2. Rabbit neutrophils (peritoneal exudate) possessed inhibitory prostanoid EP and IP receptors and a lower number of DP receptors; as observed by the low potency and comparative efficacy of PGD_2 and the selective DP receptor agonist BW 245C, against fMLP-stimulated superoxide generation. Rabbit peripheral blood neutrophils also expressed EP receptors, which appeared to be 'EP_2'-like as both AH13205 and butaprost stimulated cAMP accumulation. Further characterization as determined by sensitivity to AH 6809 antagonism, was inconclusive, as antagonism of PGE_2 was observed in only one of three rabbits.

3. Characterization of the prostanoid receptors on human peripheral blood monocytes mediating cAMP elevation suggests that monocytes possess a prostanoid EP receptor which is similar to that on human neutrophils. The EP receptor agonist (butaprost and AH13205 both active) and antagonist (BW A868C-no antagonism and AH 6809-antagonism) profiles in the human neutrophil and monocyte were similar. However the non-competitive nature of AH23848B antagonism of PGE_2 means that the presence of EP_4 receptors on human monocytes cannot be excluded. In addition to this,

HL-60 cells which can be differentiated into either neutrophilic or monocytic cells, also express functional prostanoid EP receptors with similar agonist potency orders and sensitivity to prostanoid receptor antagonists to the human neutrophil.

5.3.1 PGE_2 - an inhibitor of human neutrophil activation

PGE_2 is not a global inhibitor of human neutrophil activation. Even within a single functional assay, O_2^- generation, PGE_2 differentially inhibited human neutrophil activation by soluble stimuli, fMLP and C5a, but not particulate stimuli, opsonised zymosan (OZ). Similar selectivity was exhibited by the PDEI RO 20-1724, which attenuated fMLP but not opsonised zymosan-stimulated O_2^- generation, thus suggesting that cAMP-elevation was a common mechanism of PGE_2 and PDEIs. However, OZ-stimulated LTB_4 formation by human neutrophils was inhibited by PGE_2 and other 'EP_2'-selective agonists (Wheeldon & Vardey, 1993), with similar potency and efficacy as inhibitors of fMLP-stimulated O_2^- generation. These observations may reflect divergence of post-receptor signal transduction pathways which allows selective inhibition of responses, for instance, LTB_4 formation but not O_2^- generation, elicited by a single stimulus, OZ. Thus, modulation of neutrophil activation is determined by the way stimulatory and inhibitory signal transduction mechanisms interact.

The potentiation of OZ-stimulated O_2^- generation by sulprostone (EP_1/EP_3 selective agonist) is of interest, as Wheeldon & Vardey (1993) similarly reported sulprostone potentiated OZ-stimulated LTB_4 generation by human neutrophils. These observations suggest that pro-inflammatory EP_3 receptors are present on human neutrophils which potentiate OZ-activated neutrophil activation possibly via the MAC-1 (CD11b/CD18) integrin receptor which bind the C3bi particles of opsonised zymosan. Additional evidence to support this mechanism of action is a report by Armstrong (1992) of sulprostone and other prostanoid EP agonists with activity at EP_3 receptors stimulated neutrophil adhesion to filters in chemotaxis assays.

The modulatory effect of PGE_2 on human neutrophils is not limited to inhibition of O_2^- generation but extends to attenuation of degranulation and polarisation. PGE_2, in this study, was a potent, albeit less effective inhibitor of fMLP-stimulated β-glucuronidase release than an inhibitor of fMLP-stimulated O_2^- generation. This observation suggests a divergence of signal transduction mechanisms and sensitivity to PGE_2 inhibition in the human neutrophil controlling degranulation and NADPH-oxidase activation.

The effects of PDEIs and prostanoid EP agonists on neutrophil chemotaxis and adhesion (Harvath *et al.*, 1991, Armstrong, 1992) are more complex. In previous studies, neither type of agent was an efficacious inhibitor of fMLP-stimulated chemotaxis, and lower concentrations of EP agonists, most notably EP_3 selective agonists, stimulated neutrophil adhesion to the chemotaxis filters (Armstrong, 1992). In addition, simultaneous treatment of neutrophils with type IV selective PDEIs and EP agonists failed to potentiate inhibition of fMLP-stimulated chemotaxis (Armstrong, 1992). However, Harvath *et al.*, (1991) showed that forskolin, isoprenaline and PGE_1 were more effective inhibitors of human neutrophil chemotaxis stimulated by fMLP and LTB_4 in the presence of the non-selective PDEI IBMX.

The activation state of neutrophils in individuals with inflammatory conditions, are more likely to be primed by endogenous agents, for example, TNF_α, GMCSF and PAF (Nathan, 1987, Ho *et al.*, 1990); or activated by stimuli such as LTB_4, complement factors (C5a and C3) and chemokines (IL-8) prior to drug treatment. In this study, PGE_2 was as potent and efficacious an inhibitor of PAF-primed fMLP-stimulated O_2^- generation as it was of unprimed human neutrophils. This observation suggests that PGE_2 (or another EP receptor agonist) would be effective as an inhibitor of neutrophil activation in inflammatory conditions where an individual's neutrophils would already primed prior to exposure to PGE_2. PDEIs (Ho *et al.*, 1990) were also potent and effective inhibitors of O_2^- generation by human neutrophils primed with PAF or TNF_α.

This is as would be expected if PDEIs and PGE_2 share a similar profile of activity, as a consequence of the same transducer molecule - cAMP. However, one study reported that the ability of exogenous adenosine to inhibit asthmatics' neutrophils was diminished (Sustiel *et al.*, 1989), which suggests that in chronic inflammatory disorders, attenuation of leukocyte activation may be more difficult to achieve. However, in the same study there was little difference in PGE_2 inhibition of neutrophil activation between normals and asthmatics. Thus, a selective prostanoid EP receptor agonist may be more clinically effective in the treatment of chronic inflammatory conditions, such as asthma, than adenosine agonists.

5.3.2 Prostanoid receptors on rabbit neutrophils

The results suggest that rabbit neutrophils isolated from peripheral blood or collected from casein-induced peritoneal exudate express prostanoid receptors. As is the case with human neutrophils, the prostanoid receptors on rabbit neutrophils are not a homogenous population, but unlike human neutrophils, inhibition of O_2^- generation by rabbit neutrophils was not a suitable assay to assess the activity of prostanoids. The inhibition curves of the prostanoids in the rabbit neutrophils were shallow making interpretation of the data difficult.

PGE_2-stimulated cAMP increase in rabbit neutrophils (isolated from peripheral blood) produced better defined E/[A] curves than obtained using the O_2^- assay. The $p[A_{50}]$ values were similar to that obtained with PGE_2-stimulated cAMP increases in the human neutrophil (chapter 3) and the activity of the 'EP_2'-selective agonists butaprost and AH13205 all provide evidence of this rabbit neutrophil EP receptor resembling the EP_2 receptor. However, the inconsistent antagonism of PGE_2-stimulated increases in cAMP by AH 6809 (only one of three rabbits) may suggest that the EP receptor present on the rabbit neutrophil is an 'EP_2'-receptor similar to that described in the cat trachea (Coleman *et al.*, 1987) and is not sensitive to antagonism by AH 6809. An alternative explanation

may be that the EP receptors present on rabbit neutrophils are not a homogeneous population and that there is considerable variability in the expression of individual subtypes as exists in the rabbit jugular vein (Lawrence & Jones, 1992). The latter may explain the results presented in this present study as only a small number of animals were used and obviously warrants more work.

5.3.3 Prostanoid EP receptors on human peripheral blood monocytes

As with most early work on prostaglandins and inflammatory cells, the EP receptor on monocytes has been presumed to be the EP_2 subtype on the basis of the ability to stimulate cAMP elevation. The most detailed pharmacological characterization to date was that of Milne *et al.*, (1994), where the effect of EP receptor agonists and AH23848B (EP_4 receptor antagonist) on cAMP elevation by human monocytes was studied. They concluded that the EP agonists stimulated cAMP elevation by activating EP_4 receptors, on the basis that AH13205 (EP_2-selective agonist) was inactive and AH23848B antagonised PGE_2. An apparent pA_2 value of 5.6 was estimated from the rightward-shift of the PGE_2 curve observed in the presence of 10^{-5}M AH23848B as $3x10^{-5}$M completely abolished the PGE_2 cAMP response.

In the present study, contrary to the findings of Milne *et al.* (1994), both AH13205 and butaprost, the EP_2-selective agonists, stimulated cAMP accumulation suggesting the presence of an 'EP_2'-like receptor. Practical differences may account for the different findings presented in this study and that of Milne *et al.* The cell preparations used in the respective studies are themselves different; in the present study the monocyte preparations (90% pure) were isolated by centrifugation and the assay performed on fresh monocyte preparations (< 2 hours after final preparation) and measured total cAMP levels. However, in Milne's study, the monocyte population was prepared from a mixed mononuclear cell suspension, and the monocytes were isolated by adherence overnight. The generation of cAMP was consequently performed on adherent cells, the supernatants

removed after stimulation and only cellular cAMP assayed.

The cAMP levels measured in the monocyte suspensions in the present study with all treatments were higher than those reported by Milne *et al.*, and may therefore have been more sensitive to detect the levels of cAMP stimulated by AH13205 or butaprost which were lower than that stimulated by PGE_2. In addition to this, cAMP can be released from cells into the supernatant as has been demonstrated with human neutrophils (Harvath *et al.*, 1991). The amount released can represent a substantial proportion of the total amount of cAMP generated, for instance in the human neutrophil (Harvath *et al.*, 1991). Thus, another explanation for the different findings may be that Milne *et al.* only measured unreleased cAMP and not total cAMP generation stimulated by the EP agonists. However, the amount of cAMP retained and released by each stimulus in the monocyte may not be identical.

Yet another possible explanation is that adherent and suspended monocytes may respond differently to stimulation. The EP_4 receptor may be functionally coupled to adenylate cyclase in adherent and suspended monocytes; whilst the AH 6809-sensitive 'EP_n' receptor may only be functionally coupled to adenylate cyclase in non-adherent monocytes. A similar situation exists in human neutrophils with TNF_α which can only stimulate O_2^- generation in adherent neutrophils and not in neutrophil suspensions (Nathan, 1987).

The present studies with prostanoid DP and EP receptor antagonists suggest that PGE_2 was selectively acting at EP receptors except at the highest concentration (10^{-4}M). Selective antagonism by AH 6809 and AH23848B (10^{-5}M for both antagonists) of PGE_2-stimulated cAMP elevation was concluded on observing no antagonism of the cAMP response of the prostanoid IP receptor agonist cicaprost. AH 6809 (10^{-5}M) produced a parallel rightward displacement of the PGE_2 E/[A] curve suggesting that AH 6809 was behaving as a competitive antagonist; whilst AH 23848B (10^{-5}M) caused a

collapse of the PGE_2 curve indicating that the antagonism was non-competitive.

The antagonism by AH 6809 of PGE_2 has been observed in human neutrophils (inhibition of O_2^- generation and cAMP generation), whilst the non-competitive nature of AH23848B has been described in Jurkat cells (human T-lymphocyte cell line) (De Vries *et al.*, 1995) with a range of AH23848B concentrations. The Jurkat cells did not respond to AH13205 with an increase in cAMP levels suggesting that this human T-cell line did not possess 'EP_2'-like receptors; whilst the non-competitive depression of the PGE_2 curve by AH23848B they suggested was evidence of an atypical EP receptor. However, the non-competitive nature of AH23848B antagonism of PGE_2 has been observed in EP_4 receptor-containing tissues, such as the rabbit saphenous vein (personal communication from Simon Lydford, Astra Charnwood) indicating that non-competitive antagonism by AH23848B of PGE_2 is not inconsistent with an EP_4 receptor. Thus, the EP receptor present on Jurkat cells may actually be an EP_4 receptor, and the human monocyte possesses two EP receptors; an EP_4 receptor and an 'EP_n' receptor.

The monocyte preparations contained a significant number of platelets (1 monocyte: 2-8 platelets), however, it is unlikely that these platelets contributed significantly to the cAMP accumulation on stimulation with PGE_2. Platelets do not possess EP receptors positively coupled to adenylate cyclase and any effect of PGE_1 and PGE_2 are mediated via the prostanoid IP receptor (Myers *et al.*, 1985). Although platelets express prostanoid DP and IP receptors, platelets made only a minor contribution to the levels of cAMP generated in these preparations on stimulation with PGD_2 or cicaprost (Darius *et al.*, 1994).

5.3.4 Prostanoid EP receptors of the human promyelocytic leukaemic cell line HL-60

Undifferentiated HL-60 cells responded to the prostanoid EP agonists with an elevation of cAMP levels. The agonist profile was consistent with the EP_2 subtype and the agonist

potency order was similar to that of human peripheral blood neutrophils discussed in chapter 3. Further similarities with the neutrophil 'EP_n' receptor included AH 6809 antagonism of the PGE_2 stimulated increase in cAMP, the lack of antagonism of PGE_2 by the selective DP receptor antagonist BW A868C or the EP_1 receptor antagonist SC 19220. The location of the PGE_2 E/[A] curve in the HL-60 cells was similar to that of the human neutrophil, although the E/[A] curve was steeper in HL-60 cells (2-3 $\log_{10}$ units) than the human neutrophil (3-3.5 $\log_{10}$ units) suggesting that HL-60 cells expressed a more homogeneous population of prostanoid EP receptors. The PGE_2 superoxide inhibition E/[A] curves were also generally more shallow, suggesting that neutrophil prostanoid EP receptors were a heterogeneous population.

In a binding study recently published by Matthews *et al.* (1995) using the membranes of HL-60 cells differentiated into macrophage-like cells, showed that specific binding of [^{3}H] PGE_2 had two components. Only one of these binding sites was well-characterized as the second was not saturated by 10^{-7}M radiolabelled ligand. The higher affinity site had a K_d of 3.5×10^{-9}M, and [^{3}H] PGE_2 binding at this site was displaced by AH23848B (K_i of approximately 1.9×10^{-5}M), but not by the EP_2 selective agonists butaprost and AH13205, or the EP_1/EP_3 selective agonist sulprostone. AH 6809 (10^{-5}M) did not displace PGE_2 binding, and cicaprost, the high potency IP agonist, showed only low potency as an inhibitor of PGE_2 binding. Thus, the data from the Matthews *et al.* study suggests that the high affinity PGE_2 binding site on macrophage-like HL-60 membranes was the EP_4 receptor, and therefore undifferentiated HL-60 cells must also be able to express EP_4 receptors. However, it is evident from the location of the PGE_2 cAMP stimulation curve in undifferentiated HL-60 cells (and peripheral blood monocytes), $p[A_{50}] \approx 7.0$, that the prostanoid EP receptor responsible for the cAMP elevation is not the EP_4 subtype but is mediated via a lower affinity binding site. This would obviously brings into question the functional role of the EP_4 receptor on monocytes and

macrophage-like HL-60 cells.

Prostanoid EP agonists inhibit a variety but not all neutrophil functions, and therefore may reduce acute inflammation. Furthermore, monocyte activation may also be modulated by EP receptor agonists; as cAMP-elevating agents such as phosphodiesterase inhibitors, PGE_2 and β-adrenergic agents, inhibit monocyte function *in vitro*. This suggests that prostanoid EP agonists may modulate certain aspects of chronic inflammation which involve monocyte activation.

CHAPTER 6

GENERAL DISCUSSION

6.1 Characterization of Prostanoid Receptors

Pharmacological characterization of the prostanoid EP receptors mediating inhibition of human neutrophil activation in the present study suggests that the receptor is similar to the classical EP_2 subtype on the basis of agonists potency ratios (chapter 3). These findings are in agreement with other published studies (Wheeldon & Vardey, 1993; Talpain *et al.*, 1994). Additional evidence of the similarity of the neutrophil and the classical EP_2 subtype arose from the neutrophil cAMP studies with prostanoid EP agonists (chapter 3). The agonist potency order for elevation of cAMP of EP agonists was similar to that of inhibition of fMLP-stimulated superoxide generation; and cAMP elevation is believed to be the signal transduction pathway of EP_2 receptors (Yeardley *et al.*, 1993).

However, further characterization of the human neutrophil EP receptor with prostanoid receptor antagonists (chapter 3) showed that AH 6809 antagonised EP_2 receptor mediated responses, in contradiction to the existing classification. Antagonism by AH 6809 was observed against PGE_2-mediated inhibition of superoxide generation and PGE_2-stimulated cAMP elevation. Doubt had been cast on the specificity of AH 6809 as an antagonist in the human neutrophil (Talpain *et al.*, 1994), but the antagonism of AH 6809 by PGE_2 in the human leukocytes observed in the present study showed specificity and selectivity.

However, the degree of antagonism by AH 6809 (10^{-5}M) of PGE_2 in the human neutrophil superoxide assay using a large pool of donors was variable (chapter 3), but may be explained by the presence of heterogenous populations of prostanoid EP receptors some of which may not be sensitive to AH 6809 antagonism. The relative proportions of these receptors on neutrophils may differ between individuals as would the apparent sensitivity of PGE_2 to AH 6809 antagonism. Such a situation would result in an apparent "wall" effect as described in chapter 3, preventing AH 6809 (10^{-5}M) producing as large a rightward shift of the PGE_2

E/[A] curve as would have been predicted from the pA_2 value estimated from the early O_2^- experiments. Alternatively, the estimate of the pA_2 value for AH 6809 was conducted on relatively few donors, and may not represent the true pA_2 value, which would only be realised from using a larger population of donors.

EP receptors are the only prostanoid receptors with clear pharmacological evidence for subdivision, but rigorous pharmacological characterization of 'EP_2'-receptors in human tissues and those of other species is lacking. Therefore, the findings presented in this thesis are not conclusive evidence for the neutrophil EP receptor either being a AH 6809-sensitive human homologue of the classical EP_2-receptor or a novel prostanoid EP receptor subtype. However, the findings presented in this thesis suggest that the existing classification shown in Table 3.1 may require revision to take into account the antagonism of the human EP_2 receptor by AH 6809, termed 'EP_n', (Table 6.1). In addition, the use of AH 6809 as a selective prostanoid DP/EP_1 receptor antagonist receptor should be reevaluated as antagonism of prostanoid EP_2 receptors may be an additional property. AH 6809 antagonises prostanoid DP, EP_1 and EP_2 responses over a similar concentration range (10^{-6}M-10^{-5}M), and should be regarded as a less selective prostanoid receptor antagonist. Furthermore, AH 6809 has been omitted from the 1995 edition of the Trends in Pharmacological Studies (TIPS) annual compendium of receptor classification, suggesting that AH 6809 is no longer regarded as a selective prostanoid DP/EP_1 receptor antagonist.

However the classification to date has relied upon the use of tissues, mainly smooth muscle preparations from a range of species, such as the guinea pig ileum (EP_1), cat trachea (EP_2), guinea pig vas deferens (EP_3) and piglet saphenous vein (EP_4). Thus the characterization of prostanoid receptors in any tissue from any species is subject to a degree of uncertainty as species differences may exist in their sensitivity to different agonists and antagonists. One example of this is another prostanoid receptor, the thromboxane (TP) receptor. Most TP

receptor antagonists have 10-fold higher pA_2 value in the aorta and platelets of humans, rats and dogs compared to the corresponding rabbit tissues (Lumley *et al.*, 1989 and Tymkewycz *et al.*, 1991). Other pharmacological parameters, for instance, TP agonist potency orders were similar in human, rat, dog and rabbit tissues, suggesting that the differences in antagonist potency between rabbit and the other species' tissues were unlikely to be due to TP receptor subdivision. A more likely explanation is differences in the affinity of antagonists for TP receptors between species.

Species differences in the potency of antagonists at prostanoid EP receptors may also apply to human tissue. There is a limited supply of normal human tissue except for blood derived cells (platelets and leukocytes), which is the main difficulty associated with the classification of human prostanoid receptors. However, there are many human cell lines which may represent a useful source of human 'tissue' for pharmacological studies. One such cell line is the human promyelocytic leukaemic cell line (HL-60), in which the prostanoid EP receptor was characterized in the present study (chapter 5). HL-60 cells which may be differentiated into either neutrophil-like or monocyte-like cells, expressed prostanoid 'EP_2'-like receptors as determined by agonist potency orders. Furthermore, the HL-60 EP receptor appeared to be sensitive to AH 6809 antagonism (chapter 5), as described in the human neutrophil suggesting it is the 'EP_n' receptor (chapter 3).

The presence of a AH 6809-sensitive 'EP_2'-like receptor mediating cAMP elevation was also demonstrated in human peripheral blood monocytes (chapter 5) suggesting that the 'EP_n' receptor is present on many human leukocytes. Whether the same EP receptor subtype is expressed by other human leukocytes, such as eosinophils and lymphocytes remains to be discovered. Knowledge of the distribution of the 'EP_n' receptor would throw light on the immunomodulatory and anti-inflammatory potential of selective agonists for this receptor for the treatment of acute and chronic inflammation.

Prostanoid EP receptor classification is however impeded by a lack of selective and potent antagonists for the receptor subtypes. It is clear from the experiments with AH 6809 and AH23848B in the human neutrophil, that both EP receptor antagonists have other activities in leukocytes not directly associated with their prostanoid receptor antagonist properties (chapter 3; Talpain *et al.*, 1994). These activities interfere with leukocyte functional assays such as superoxide generation, and have limited their use as antagonists and hampered the interpretation of their antagonism of prostanoid agonists.

In view of the less than ideal potency and selectivity of prostanoid EP receptor agonists and antagonists, as well as the additional problems of human prostanoid receptor classification, additional evidence with respect to receptor classification may be provided by recent advances in molecular biology. The first human prostanoid TP receptor was cloned (Hirata *et al.*, 1991) from cDNA libraries of cultured megakaryocyte leukaemia cells and human placenta obtained from partial amino acid sequences of purified protein from the membranes of human platelets. The murine prostanoid TP receptor was then cloned (Namba *et al.*, 1992) and used as a hybridisation probe to clone the murine EP_3 receptor by homology screening (Sugimoto *et al.*, 1992). Subsequently murine (Honda *et al.*, 1993), rat (Sando *et al.*, 1994), and human (An *et al.*, 1993, Bastien *et al.*, 1994) 'EP_2' receptors were also cloned.

The cloned murine EP receptor which was initially designated the 'EP_2' subtype on the basis that PGE_2 stimulated a cAMP increase (Honda *et al.*, 1993), was consequently re-assigned the EP_4 notation. The basis for this was the observation that butaprost, the EP_2 selective receptor agonist, did not displace PGE_2 binding of the cloned receptor expressed in COS cells (Nishigaki *et al.*, 1995). This illustrates that demonstration of a functional response mediated by a transfected cloned receptor is insufficient confirmational evidence of receptor identity, and that pharmacological characterization and evaluation of functional data is essential. Many publications detailing the successful cloning of prostanoid receptors lack

pharmacological evaluation of the functional agonist and antagonist data, thus precluding confirmation of the transfected (cloned) receptor as being identical to the naturally-occurring receptor which was the target of the initial cloning strategy.

Prostanoid receptors are expressed by most if not all mammalian cell types, as would have been expected from the ubiquitous distribution of prostaglandins throughout the body. Consequently finding a suitable mammalian cell line which does not constitutively express prostanoid receptors in which to transfect cloned prostanoid receptors has been difficult. Even if this is achieved, there is no guarantee that the transfected receptor will be expressed in the same way, for instance differences in the folding of the protein and glycosylation patterns. A functional response may not be detectable, as the transducer molecules may be different, for instance G protein types and numbers or different isoforms of adenylate cyclase may differ. In addition, alternative splice variants at the carboxy-terminal of transfected cloned bovine (Namba *et al.*, 1993), human (Regan *et al.*, 1994b), murine (Sugimoto *et al.*, 1993) and rabbit (Breyer *et al.*, 1994) prostanoid EP_3 subtypes have been identified. Normally EP_3 receptors are negatively coupled to adenylate cyclase via G_i, however some alternative splice variants were able to couple to G_s instead of G_i and activated adenylate cyclase, but all the splice variants exhibit similar agonist potency orders for binding. However, no splice variants have been found for any of the other EP receptor subtypes, TP or FP receptors. The constitutive expression of prostanoid receptor splice variants in normal cells is unclear, as is their physiological significance in dictating tissue responses to agonists and antagonists.

Cloned receptors have been successfully transfected into non-mammalian cells such as *Xenopus* oocytes (human TP receptors, Hirata *et al.*, 1991), bacteria such as *Escherishia coli* (E. coli) and insect cell lines e.g. *Spodoptera frugiperda* (Sf-9) cells. The stable transfection of adenylate cyclase linked β-adrenergic receptors into insect Sf-9 cells has been achieved

(Mouillac *et al.*, 1992, Kleymann *et al.*, 1993, Ravet *et al.*, 1993), overcoming the problems of phylogenic differences. These insect cells may be prove to be suitable for transfection with prostanoid receptors such as those identified in human neutrophils and monocytes. Cloned prostanoid EP_3 receptors have also been successfully transiently transfected into insect Trichoplusia ni (MG1) cells (Huang & Tai, 1994). These reports provide evidence that it may be feasible transfect cloned prostanoid EP receptors which normally couple to adenylate cyclase into Sf-9 cells.

The human prostanoid 'EP_2' receptor, Hup-4, cloned from human placenta, when transfected into COS-7 cells was found to be positively coupled to adenylate cyclase. The pharmacological prostanoid agonist profile in binding studies (displacing ^{125}I labelled PGE_2 binding) and functional studies (cAMP generation) was consistent with the 'EP_2' receptor (Regan *et al.*, 1994a). As no antagonists were tested in the Hup-4 transfected cells, it is unclear whether Hup-4 is the 'EP_n' receptor characterized in the human neutrophil. Northern blotting has shown that Hup-4 is present in HL-60 cells, and as PGE_2-stimulated cAMP accumulation is AH 6809-sensitive in undifferentiated HL-60 cells (chapter 5), suggesting that 'EP_n' and Hup-4 are the same receptor. The only proof would come from blocking the expression of Hup-4 in HL-60 cells, for example by the use of the antisense approach and confirming this at the message and protein level, then using these modified cells in experiments to evaluate the effect of AH 6809 on PGE_2. In addition, AH 6809 antagonises PGE_2 binding to Hup-4 and PGE_2-stimulated cAMP generation in Hup-4 transfected COS-7 cells (Woodward, personal communication). The results presented in this thesis on the EP-receptor pharmacology of human neutrophils and HL-60 cells support such speculation. Furthermore, the pharmacology of the human monocyte 'EP_2-like' receptor is also similar to that of the human neutrophil and HL-60 cells (which can be differentiated into monocyte-like cells or neutrophil-like cells).

The Hup-4 amino acid sequence is distinctly different to the other two human 'EP_2' receptor clones (An *et al.*, 1993 and Bastien *et al.*, 1994). However, the degree of homology between them and Hup-4 was similar to that between Hup-4 and human EP_1 or EP_3 receptors. In contrast, there was close homology between these human 'EP_2' receptors and the human EP_1 or EP_3 receptors (~80%) and the murine 'EP_2' and renamed EP_4 receptor. The receptors were cloned from human peripheral blood polymorphonuclear leukocytes and a human lung cDNA library respectively using reverse transcription and with cDNA primers prepared from mouse EP_3 and human thromboxane A_2 receptors (An *et al.*, 1993) and the murine EP_2 receptor sequences (Bastien *et al.*, 1994). The amino acid sequence between these two clones as almost identical as was the distribution of their mRNA expression in human tissue, i.e. strongly expressed in lung, kidney, small intestine and thymus. The pharmacology of these two 'EP_2' receptors is different to Hup-4. PGE_2 stimulated cAMP generation via the transfected 'EP_2' receptors (An *et al.*, 1993 and Bastien *et al.*, 1994), but butaprost was unable to displace radio-labelled PGE_2 binding to the same transfected EP_2 receptors (Bastien *et al.*, 1994) which is not consistent with the pharmacology of the classical EP_2 receptor. As would have been predicted from the lack of pharmacological analysis in these studies, the designation of the 'EP_2' notation was premature as the limited pharmacology presented in those studies suggest that they may actually represent the EP_4 subtype.

The expression of these two human 'EP_2' receptor clones in thymus, spleen and leukocytes, and the fact that An *et al.* (1993) used polymorphonuclear leukocytes, which are predominately neutrophils, to generate the cDNA primers, would suggest that there may be EP_4 receptors expressed by these leukocytes. However, the pharmacology of PGE_2 and other prostanoid EP receptor agonists and antagonists in human neutrophils does not reflect the presence of an EP_4 receptor. Possible explanations may be the comparatively low

expression of EP_4 receptors by human neutrophils compared to the other EP receptors present, or that EP_4 receptors are only weakly coupled to effector mechanisms. An alternative explanation is that the stringency of the primers used may have favoured the detection and isolation of the EP_4 receptor from either neutrophils or possibly other contaminating leukocytes in the polymorphonuclear cell preparation such as monocytes, lymphocytes or eosinophils. The study of An *et al.* (1993) did not report the purity of the cell preparation, in which case the detection of EP_4 in the mRNA of peripheral blood leukocytes may reflect its presence in other leukocytes. As human monocytes express EP_4 receptors (chapter 5; Milne *et al.* (1994)), monocytes may be the source of the EP_4 receptor mRNA. In which case, the cloning of the EP_4 receptor from a human polymorphonuclear cell preparation by An *et al.* (1993) may have been mistakenly interpreted as the neutrophil EP_2 receptor.

However, only the demonstration of Hup-4 expression by human neutrophils and monocytes, and AH 6809 antagonism of PGE_2 binding and/or cAMP generation mediated via the transfected Hup-4 receptor, would conclusively show that Hup-4 was identical to 'EP_n'. Hup-4 is present in many tissues, for instance, the placenta, and knowledge of its distribution in additional human tissues would allude to possible *in vivo* effects of agonists for this receptor. Thus, it may be feasible to synthesise agonists for the Hup-4 receptor with selective anti-inflammatory effect on neutrophils and monocytes. Not only would such agonists represent a novel anti-inflammatory strategy, but lead to the re-evaluation of the classification of prostanoid EP receptors.

Receptor Subtype	Selective Agonist	Selective Antagonist	pA_2
EP_1	Sulprostone	SC 19220 ¶AH 6809	5.2-5.6 6.4-7.0
EP_2	AH13205/Butaprost	None	
↕ the same?			
'EP_n'	**AH13205/Butaprost**	**AH 6809**	**6.3-7.0**
EP_3	Sulprostone/GR63799X	None	
EP_4	None	AH23848B	5.4

Table 6.1 Modification of the existing classification of prostanoid EP receptor subtypes, selective agonists and antagonists. The data presented as shown in Table 3.1 with the inclusion of the prostanoid EP receptor data from this thesis shown as the 'EP_n' subtype. ¶AH 6809 is also a prostanoid DP receptor antagonist (see Table 1.1).

6.2 Prostanoid EP receptors: effects on leukocyte intracellular signalling and activation

Although it is generally accepted that prostanoid EP receptor-mediated inhibition of leukocyte activation utilizes cAMP as a transducer molecule, the precise mechanism of action is not well understood. The effects of PGE_2 and PDEIs on neutrophil activation, cAMP elevation and $[Ca^{2+}]_i$ presented in this thesis (chapter 4) suggest that there are multiple sites of interaction between these two signal transducer molecules. Consequently, interpretation of the effects of cAMP elevation and changes in $[Ca^{2+}]_i$ on neutrophil activation is not easy. These difficulties reflect the limitations of the methods used to determine the effects of inhibitors and stimuli of cell activation on cAMP levels and $[Ca^{2+}]_i$.

cAMP levels in this present study, as in most studies, were measured in the presence of a phosphodiesterase inhibitor and/or at particular time points and therefore do not reflect temporal or regional changes in cellular cAMP levels which accompany cell activation. Compartmentalisation of cellular cAMP has been postulated in human neutrophils (Bourne, 1972), and demonstrated in other cell types by Barsony & Marx (1990) with E-type prostaglandins, isoprenaline and PDEIs. These agents stimulated intracellular localised cAMP increases which changed with time; cAMP levels were initially increased in the periphery close to the plasma membrane; and gradually the fluorescence became more widespread within the cell. Forskolin, in contrast, showed a different distribution of cellular cAMP increase; initial increases were found to be perinuclear. These regional increases in cAMP suggest that the receptor-activated adenylate cyclase is closely associated with the plasma membrane, as is protein kinase AII (ubiquitous) and the type IV phosphodiesterase (reviewed by Houslay *et al.*, 1995). In human neutrophils and HL-60 cells, forskolin was a poor stimulus of cAMP elevation compared to PGE_2 (chapter 4 and 5 respectively). Forskolin was also a weak inhibitor of fMLP-stimulated O_2^- generation in human neutrophils compared to PGE_2. The difference between agonist-receptor stimulated increases in cAMP

and those induced by forskolin may reflect the differential ability of forskolin to stimulate different isoforms of adenylate cyclase. Forskolin may preferentially activate cytosolic or perinuclear adenylate cyclases rather than plasma membrane bound isoforms.

An additional complicating factor is that some of the cAMP generated is extruded from the cell and found extracellularly (Bourne, 1972 and Harvath *et al.*, 1991). This may represent another mechanism employed by the neutrophil to regulate perimembrane cAMP levels in addition to the activity of phosphodiesterases. The extrusion mechanism involved has not been identified and the physiological relevance of the extruded cAMP puzzling as cAMP is membrane impermeable and all targets for cAMP are intracellular. Comparison of the effects of neutrophil stimuli and inhibitors on cellular cAMP by measuring total cAMP levels may be subject to compartmentalisation and extrusion of cAMP and may not correlate well with their effects on neutrophil activation.

The type IV PDE isoforms found in human neutrophils were A2, A4 and A7, the former is located at the plasma membrane. Houslay *et al.* (1995) proposed that type IV PDEs were located close to other members of the cAMP modulatory pathway such as adenylate cyclase, PKA and AKAP (type A kinase anchoring protein), to compartmentalise and localise cAMP increases to certain regions. In the neutrophil, cAMP may regulate cell activation by modulating the function of plasma membrane Ca^{2+} pumps, NADPH oxidase, secretory granules and cytoskeletal components and adhesion molecules involved in chemotaxis and adhesion.

The perimembrane localisation of the components of the cAMP system in the neutrophil are well placed to interact with components of the Ca^{2+} system such as Ca^{2+} channels through which Ca^{2+} influx occurs from the extracellular medium and the plasma membrane Ca^{2+} pumps promoting the extrusion of cytosolic Ca^{2+} ions. The function of the Rap1A and cytochrome b_{558} components of the active NADPH oxidase are affected by cAMP-mediated

phosphorylation, are also closely associated with the plasma membrane (see general introduction, chapter 1).

Thus, cAMP and Ca^{2+} and NADPH oxidase; the signal transducer pathways and functional response of the neutrophil studied in this thesis, are physically in close proximity and perfectly placed for intimate interactions (Fig 6.1).

The distribution of SERCA2b, the cAMP-dependent phospholamban-activated intracellular Ca^{2+}ATPase, is closely associated with the F-actin in activated human neutrophils and thereby well-placed to regulate $[Ca^{2+}]_i$ at sites of phagocytosis (Stendahl *et al.*, 1994). Downey *et al.* (1991) showed that neutrophil microfilament assembly is sensitive to modulation by cAMP and F-actin polymerisation is required for chemotaxis, shape change and adhesion. SERCA2b may also be localised at sites of pseudopodia formation and adhesion where focal increases in $[Ca^{2+}]_i$ are also required. Stimulation of SERCA2b by cAMP may contribute to PGE_2 and PDEI mediated inhibition of OZ-stimulated superoxide generation by human neutrophils (chapter 5). Neutrophil stimulation by OZ requires phagocytosis of OZ mediated by binding the C3bi (MAC1 integrin) receptor, which also mediates neutrophil adhesion. Stimulation of SERCA2b by cAMP would attenuate the rise in $[Ca^{2+}]_i$ required for these functions as would other cAMP-dependent mechanisms which decrease $[Ca^{2+}]_i$ already described. These include the stimulation of plasma membrane Ca^{2+} pumps such as the cAMP-stimulated plasma membrane Ca^{2+} pumps are present in human platelets (Johansson *et al.*, 1992), erythrocytes (James *et al.*, 1989) and smooth muscle (Khan & Grover, 1991). PGE_2 and PDEI attenuation of fMLP-stimulated increase in $[Ca^{2+}]_i$ by accelerating the decay of the Ca^{2+} transient and reducing the magnitude of the protracted influx-dependent increase in $[Ca^{2+}]_i$ (chapter 4) may be mediated either or both of these mechanisms. Furthermore, human neutrophil nonselective cation channels are regulated by

cAMP (Schumann *et al.*, 1992) as is Ca^{2+} and Mn^{2+} influx as observed in this present study (chapter 4) suggesting that cAMP does indeed regulate ion channel function.

Cross-talk not only exists between cAMP and Ca^{2+}, but also occurs between cAMP and other classical signal transducer molecules for instance protein kinase C (reviewed by Houslay, 1991). NADPH-oxidase function may be regulated by protein kinase C (Tauber, 1987) and cAMP (via Rap1A), as may $[Ca^{2+}]_i$ as the plasma membrane Ca^{2+} pump is activated by phorbol esters (Lagast *et al.*, 1984b) and fMLP-stimulated increase in $[Ca^{2+}]_i$ is attenuated by cAMP-elevating agents as has been shown in this thesis (chapter 4) and by others.

As the cAMP-dependent protein kinase phosphorylates Rap1A, a member of the more recently discovered group of low-molecular-weight GTPases (LMWG), cAMP may also interact with LMWG such as the Rac2 protein (Knaus *et al.*, 1991) which has been identified as a regulatory protein of NADPH-oxidase. As Rac and other LMWG also regulate cytoskeletal changes (reviewed by Bokoch, 1995), this represents another possible site of interaction with cAMP. PGE_2-mediated inhibition of IL-8 and fMLP-stimulated human neutrophil polarisation (chapter 5), a response which involves cytoskeletal rearrangement, may involve such a mechanism. As chemotaxis is not dependent on extracellular Ca^{2+} ions (Merritt *et al.*, 1991), the effects of cAMP elevating agents (Harvath *et al.*, 1991) may also implicate a similar LMWG mechanism.

Other signal transduction pathways involved in neutrophil activation are beginning to be elucidated, for instance, phosphatidylinositol 3-kinase (PI3K) and tyrosine kinases. How these pathways interact with cAMP and other classical signal transducer molecules, such as Ca^{2+}, warrants further investigation.

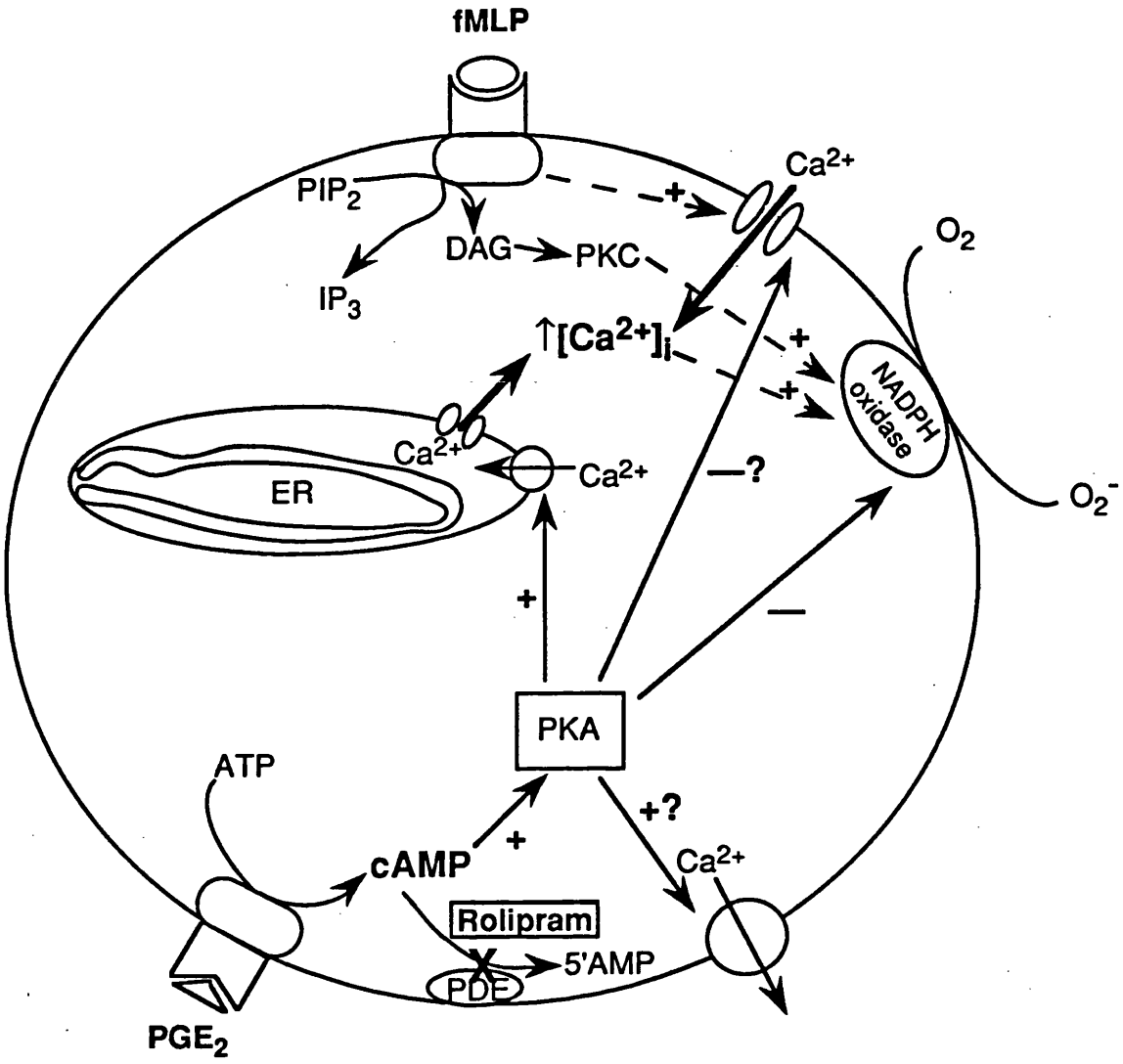


Fig 6.1 **Schematic illustration of the possible mechanisms of action of cAMP-elevating agents regulating $[Ca^{2+}]_i$ and superoxide generation in the human neutrophil.**

6.3 Prostanoid EP receptor agonist-mediated inhibition of human leukocyte activation: implications to acute and chronic inflammation

In the present study the leukocytes responses to stimuli such as fMLP, and inhibitors like prostaglandins, were variable between donors (chapter 3). These observations highlight the versatile nature and role of these leukocytes in host defence, which is to respond rapidly and effectively to the constantly fluctuating demands of the immune system. Leukocytes must protect the body from all types of insult, in any part of the body, whether it be local or systemic to eliminate the injurious agent, limit tissue damage and initiate repair. However, the immunological status of an individual is constantly changing, so even one leukocyte phenotype from an individual donor taken at different times may respond differently. Neutrophils undergo rapid turnover (circulating $t_{1/2}$ 12 hours, McAfee *et al.*, 1976), which is an inherent variable to aid the rapid reaction and adaptability of the immune system to ever changing demands.

It is not surprising perhaps that the effects of prostanoid agonists and antagonists on leukocyte functional assays are more variable than pharmacologists would like to see and expect. However, much *in vitro* pharmacological research is based upon work performed on isolated tissues obtained from genetically constrained strains of animals kept in tightly controlled environments, and so may give a misleadingly high expectation of the consistency of agonist effects in tissues which from less controlled sources. As humans are different, their inherent variability may be the reason for the range of drug efficacies in the clinic, and why some individuals are more susceptible to certain side effects than others.

A range of cell types, such as epithelial cells (Churchill *et al.*, 1989), fibroblasts (Carley *et al.*, 1992), monocytes/macrophages (MacDermot *et al.*, 1984) and smooth muscle cells (Haye-Legrand *et al.*, 1986) are capable of releasing PGE_2 which inhibits human leukocyte activation. Such a mechanism may physiologically modulate leukocyte function preventing

inappropriate or excessive activation. These cell types are located in areas where leukocytes will come into close contact during normal trafficking and during inflammation. The local synthesis of prostaglandins may suppress the activation state of the leukocyte to prevent damage to the host tissue as a self-protective mechanism. Tissue monocytes/macrophages release chemotactic agents such as IL-8 in addition to PGE_2; and may regulate neutrophil infiltration and activation in inflammatory sites by the release of such cocktails of neutrophil stimulatory and inhibitory agents. Synovial cells also have the capacity to synthesise and release chemokines, IL-8 for example (Watson *et al.*, 1988a and b) and PGE_2 (McGuire-Goldring *et al.*, 1983) and may serve a similar role in inflammation of the joint.

Misoprostol is one of the few prostanoid EP agonists which has been used clinically. Rheumatoid arthritis patients treated with NSAIDs are prone to gastric ulceration, and misoprostol has been used in conjunction with NSAIDs to prevent NSAID-induced gastropathy (reviewed by Garris & Kirkwood, 1989). Misoprostol was as effective as histamine H_2 receptor antagonists in the prevention of gastric ulceration, but increased the incidence of diarrhoea 3 fold. Part of the efficacy of misoprostol may be due to restoration of the gut cytoprotective effect of endogenous prostaglandins abolished by NSAID treatment. Leukocyte adherence is believed to play a role in the pathogenesis of NSAID-induced ulceration (Wallace *et al.*, 1991), and misoprostol by inhibiting leukocyte adhesion may attenuate the accumulation of leukocyte at ulcerogenic sites. However, the results of the misoprostol/NSAID cotreatment clinical trial suggested that misoprostol had no advantage over established anti-ulcer therapy, and had undesirable side effects in a high proportion of people. In addition, as prostaglandins are abortifacients the use of misoprostol is contraindicated in women of child-bearing age. The problems observed in these patients may be due to the lack of selectivity of misoprostol as an EP receptor agonist, which has similar potency at prostanoid EP_2/EP_3 and EP_4 receptors (Coleman *et al.*, 1994b). However, the development of more selective prostanoid EP receptor agonists may overcome such

problems, and be useful for the treatment of gastro-intestinal inflammation and other inflammatory conditions such as asthma, ARDS, arthritis, and psoriasis.

Orally administered misoprostol has been shown to inhibit neutrophil, eosinophil and monocyte accumulation in allergic cutaneous inflammation induced in atopic volunteers (Alam *et al.*, 1993), suggesting that inhibitory prostanoid EP receptors are present in these leukocytes. Misoprostol selectively inhibited the late (leukocyte infiltration-dependent) but not early inflammatory response. Leukocytes from misoprostol-treated individuals showed a reduction in leukocyte activation (eosinophils and lymphocytes) (Alam *et al.*, 1993), with a decrease in eosinophil survival in culture and decrease in GMCSF generation by lymphocytes. Systemically administered prostaglandins may therefore be therapeutically beneficial for the treatment of allergic inflammatory diseases such as asthma and also psoriasis and other inflammatory skin conditions.

Inhaled prostanoid agonists may also be beneficial for the treatment of inflammation of the airway. Inhaled PGE_2 inhibited the early and late phases of allergen-induced bronchoconstriction and hyperreactivity in human asthmatics (Pavord *et al.*, 1992), suggesting that lung inflammation may represent another therapeutic target for selective EP agonists. AH13205, the prostanoid EP_2 receptor agonist, however produced no relief from bronchoconstriction in humans and induced cough (Nials *et al.*, 1993). The lack of potency of AH13205 as an EP receptor agonist further highlights the need for more potent selective agonists for EP_2 and other relaxant EP receptors to evaluate the antiinflammatory effects of prostanoid EP agonists in the lung and other inflammatory sites. Furthermore, the characterization of the prostanoid receptors which mediate cough warrants further investigation as it is an undesirable effect of EP agonists which may be avoided if cough and bronchodilation are mediated by different receptors.

The anti-inflammatory effects of prostanoid EP receptor agonists involving inhibition of

leukocyte activation could be useful in the treatment of inflammatory disease. Indeed, Gordon *et al.* (1976) showed that lymphocyte activation was inhibited by local PGE_2, which was the earliest demonstration of an anti-inflammatory effect of a prostanoid EP receptor agonist. However the use of prostanoid agonists in humans is limited by their lack of selectivity, such as the diarrhoeagenic activity and abortifacient potential of misoprostol, and the tussive effect of AH13205. These problems can only be overcome by the development of more selective agonists for prostanoid EP receptors which mediate inhibition of leukocyte activation. This, in turn, depends on the characterization of these leukocyte receptors which has been attempted in the study presented in this thesis. Further progress can only be achieved by the combining molecular biology and pharmacological techniques to characterize different receptor subtypes, their distribution and their biological effects.

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PUBLICATIONS RELATING TO THIS THESIS

ABSTRACTS

Li, S.W., Boughton-Smith, N.K., McKechnie, K., Blackham, A. & Leff, P. (1993). Inhibition by EP receptor agonists of formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated neutrophil superoxide production and relationship to increases in cAMP. *Br. J. Pharmacol.*, **109**, 1P.

Li, S.W., McKechnie, K.C.W., Westwick, J. & Boughton-Smith, N.K. (1994). Comparison of prostanoid EP agonist responses in human neutrophils and HL-60 cells. *IX International Conference on Prostaglandins and Related Substances, 6-10th June 1994, Florence.* P84.

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Li, S.W., McKechnie, K.C.W., Griffiths, R.J., Boughton-Smith, N.K., Blackham, A. & Leff, P. (1995). Prostanoid antagonist AH 6809 antagonises PGE_2 inhibition of human neutrophil activation. *Br. J. Pharmacol.*, **114**, 82P.

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PUBLICATIONS

Li, S.W., Boughton-Smith, N.K. & Westwick, J. (1995). cAMP-elevating agents inhibit fMLP-stimulated Ca^{2+} and Mn^{2+} entry but not mobilisation in human neutrophils. (manuscript in preparation).

Inhibition by prostanoid EP receptor agonists of formyl-methionyl-leucyl-phenylalanine (FMLP) stimulated neutrophil superoxide production and relationship to increases in cAMP

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Prostaglandins of the E series suppress a variety of neutrophil functions, such as superoxide production by a mechanism that involves increases in cAMP formation (Hecker *et al.*, 1990; Ney & Schrör, 1991). However, the coupling between cAMP and neutrophil modulation has not been analysed pharmacologically, either to verify whether the two functional responses are mediated by the same receptor, or to determine the quantitative relationship between them. In this study we have used PGE_2 and some other EP receptor agonists to define this relationship.

Human neutrophils were isolated from venous blood by centrifugation with Polymorphprep (Nycomed). Neutrophils (10^6 cells ml^{-1}) were preincubated (37°C) with cytochalasin B (5μg ml^{-1}) and prostanoids or vehicle (≤ 0.25% ethanol) for 5 min in phosphate buffered saline (containing 0.9mM $CaCl_2$, 0.5mM $MgCl_2$ and 11mM glucose) prior to stimulation with FMLP (submaximal concentration, 10^{-7}M) for 5 min at 37°C. Superoxide production was measured as the reduction of cytochrome C (0.08mM, A550nm). The cAMP generation was determined from $2x10^7$ cells ml^{-1} pretreated for 5 min with isobutylmethyl xanthine (500μM) and stimulated for 5 min with the EP receptor agonists or vehicle (≤ 0.25% ethanol). After addition of perchloric acid (5 % final concentration, 4°C) and centrifugation (30s at 10,000g) cAMP was extracted using tri-n-octylamine/Freon, and assayed by ^{3}H-RIA (Amersham).

All the agonists tested behaved as full agonists against FMLP (10^{-7} M) stimulated superoxide production (9.9 ± 0.5nmol 10^6 $cells^{-1}$) whereas two of the agonists, PGA_1 and AH 13205, behaved as partial agonists of cAMP formation. α and pEC_{50} values obtained by fitting a logistic equation to concentration-effect data are shown in the Table 1.

Table 1

pEC_{50} (and α values for cAMP) for PGE_2, 11-deoxy PGE_1, misoprostol, PGA_1 and AH 13205 in superoxide and cAMP assays

Agonist	PGE_2	11-deoxy PGE_1	Misoprostol	PGA_1	AH 13205
Superoxide	7.2±0.1 (n=30)	6.1±0.2 (n=7)	6.3±0.4 (n=3)	6.3±0.2 (n=3)	5.5±0.2 (n=7)
cAMP	6.8±0.1 (α =1, n=21)	5.6±0.2 (α =1, n=4)	5.2 (α =1, n=4)	6.3±0.2 (α =0.62, n=3)	5.8±0.1 (α =0.34, n=3)

Analysis of the two sets of data using operational model-fitting (Leff *et al.*, 1990) showed that they differ only in terms of the receptor reserve operating in the two assays. It is concluded, therefore, that the receptors subserving cAMP elevation and superoxide inhibition are the same. Additionally, affinity and efficacy values could be estimated for PGA_1 and AH 13205 demonstrating that cAMP measurement provides a more quantitative analysis of agonist effects than inhibition of superoxide production.

AH 13205 and misoprostol were gifts from Glaxo and Searle respectively.

Hecker, G., Ney, P. & Schrör, K. (1990). *Naunyn - Schmiedeberg's Arch Pharmacol.*, 341, 308 - 315.
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COMPARISON OF PROSTANOID EP AGONIST cAMP RESPONSES IN HUMAN NEUTROPHILS AND HL-60 CELLS

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Prostanoid EP receptor agonists inhibit FMLP stimulated superoxide generation and increase cAMP in human neutrophils (Li *et al.*, 1993). Human promyelocytic leukaemic HL-60 cells can be differentiated to become neutrophil-like and appear to express EP_2 receptors (Armstrong & Talpain, 1992, Hollingsworth & De Vries, 1992). This study compares cAMP responses of EP agonists in undifferentiated HL-60s and human neutrophils to determine whether they are mediated by the same receptor subtype. The HL-60s when stimulated with PGE_2 ($p[A_{50}]$ = 6.8±0.1, n=5) generated 30-50 fold more cAMP than human neutrophils ($p[A_{50}]$ = 6.8±0.1, n=21). The EP agonists; 11-deoxy PGE_1, Misoprostol, PGA_1 and AH13205 stimulated cAMP production in HL-60s (mean $p[A_{50}]$ values were 5.7±0.1, 5.5±0.1, 5.7±0.1 and 5.5±0.1 respectively, n=4-5) and human neutrophils (mean $p[A_{50}]$ values were 5.6±0.2, 5.2±0.2, 6.3±0.2 and 5.8±0.1 respectively, n=3-4) but did not all reach the same maximum response in both cell types. Analysis of the two sets of data using operational model fitting (Leff *et al.*, 1990) showed that the same agonist potency order existed in both cell types i.e. PGE_2 > 11-deoxy $PGE_1 \approx PGA_1$ > Misoprostol ≥ AH13205. Therefore these data suggest that the same EP receptor subserves cAMP elevation in human neutrophils and undifferentiated HL-60 cells.

AH13205 and Misoprostol were gifts from Glaxo and Searle respectively.

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cAMP-elevating agents inhibit Ca^{2+} entry but not mobilisation in human neutrophils

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Prostaglandin E_2 (PGE_2) (Hecker *et al.*, 1990) and selective type IV phosphodiesterase inhibitors (PDEI's), such as rolipram (Schudt *et al.*, 1991), inhibit FMLP-stimulated superoxide (O_2^-) generation by human neutrophils. FMLP-stimulated increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in human neutrophils is also inhibited by PGE_2 and rolipram. Both agents elevate cAMP levels, PGE_2 by activating adenylate cyclase which catalyses the formation of cAMP from ATP, and PDEI's by inhibiting cAMP metabolism. It is unclear whether cAMP-elevating agents inhibit human neutrophil activation by attenuating $[Ca^{2+}]_i$ and if they do, whether this is mediated by modulating Ca^{2+}-mobilisation and/or entry.

In this study, we investigated the effect of cAMP elevation on Ca^{2+} release from intracellular stores and Ca^{2+} influx in fura-2 loaded human neutrophils ($5x10^6$ ml^{-1}) by measuring $[Ca^{2+}]_i$ and Mn^{2+} (a Ca^{2+} surrogate) influx. Chelation of extracellular Ca^{2+} with EGTA (1mM) inhibited FMLP (0.1μM)-stimulated O_2^- generation by human neutrophils (62.1±3.8%, n=3), as did PGE_2 (10μM, 89.2±3.2%, n=4) and rolipram (1μM, 70.7±3.0%, n=4). These maximally inhibitory concentrations of PGE_2 and rolipram did not reduce the FMLP (0.1μM)-induced peak increase in $[Ca^{2+}]_i$ (release from intracellular stores) but reduced the duration of the increase in $[Ca^{2+}]_i$ (influx from extracellular medium). Neither PGE_2 nor rolipram inhibited the FMLP peak Ca^{2+} transient when extracellular Ca^{2+} ions were chelated with EGTA (1mM), but the increase in $[Ca^{2+}]_i$ on re-addition of 2mM $CaCl_2$ (Ca^{2+} influx) was inhibited by PGE_2 (10μM, by 77.7±11.3%, n=3) and rolipram (1μM, by 72.8±6.3%, n=3). However Mn^{2+} influx stimulated by FMLP (0.1μM) was unaffected by either agent, and there was no measurable FMLP-stimulated Ba^{2+} (another Ca^{2+} surrogate) influx over unstimulated levels.

These data demonstrate that cAMP-elevating agents, such as PDEI's and PGE_2, inhibit FMLP-stimulated increase in $[Ca^{2+}]_i$ and provides evidence for this as a mechanism of inhibiting human neutrophil activation. This is mediated by inhibiting Ca^{2+}-entry and/or promoting Ca^{2+}-efflux/sequestration. In addition, Ca^{2+} and Mn^{2+} permeable ion channels may be differentially regulated by increases in cAMP. It will be interesting to determine whether thapsigargin-induced superoxide generation, Ca^{2+} and Mn^{2+} influx are similarly modulated by cAMP-elevating agents.

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Prostanoid antagonist AH 6809 antagonises PGE_2 inhibition of human neutrophil activation

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The potency order of prostaglandin E (EP) receptor agonists and activity of AH13205 (EP_2 selective agonist) on neutrophil superoxide production and cAMP levels, suggest the EP receptors on human neutrophils are of the EP_2 subtype (Wheeldon & Vardey, 1993 and Li *et al.*, 1993). There are, however, no selective antagonists for the EP_2 receptor. AH 6809 has been described as an antagonist for EP_1 and DP receptors (pA_2 values of 6.6-7.0, Coleman *et al.*, 1987, and 5.3, Keery & Lumley, 1988, respectively) and it has been stated that it is a weak or inactive antagonist ($pA_2 < 5.0$) at EP_2 receptors in the cat trachea and chick ileum (Coleman *et al.*, 1985). In the present study we have evaluated AH 6809 as an antagonist of PGE_2 in human neutrophils and compared it to other EP_1 (SC 19220, pA_2=5.4-5.7, Coleman *et al.*, 1987) and DP (BW A868C, pA_2=9.26, Giles *et al.*, 1993) receptor antagonists.

Human neutrophils were isolated from venous blood by centrifugation (450-470 g) with Polymorphprep (Nycomed) and pre-incubated with antagonists at room temperature (60 min) in phosphate buffered saline containing 0.9mM $CaCl_2$, 0.5mM $MgCl_2$ and 11mM glucose. Cytochalasin B (5μg ml^{-1}) and the agonists or vehicle were added and incubation continued for a further 5 min (37°C). Superoxide production (5 min, 37°C) was stimulated by the addition of FMLP (10^{-7} M) and measured as superoxide dismutase (90 U ml^{-1}) inhibitable reduction of ferri-cytochrome C (0.08mM). PGE_2 inhibition of FMLP stimulated superoxide production (pEC_{50}=6.9 ± 0.1, mean ± s.e.mean, n=12) was antagonised by AH 6809 (pA_2=7.04±0.06, slope=1.06±0.07, n=5 experiments) but not by SC 19220 (10^{-4} M). BW A868C (10^{-7} M) also failed to antagonise PGE_2, a concentration which caused a 2.38±0.09 log_{10} unit rightward-shift (mean ± s.e.mean, n=5) of the PGD_2 concentration-effect curve. Furthermore, the shift of the PGE_2 curve by AH 6809 (10^{-5} M) was unaffected by the presence of BW A868C (10^{-7} M, n=3). AH 6809 (10^{-5} M) did not antagonise inhibition of superoxide production mediated by the adenosine receptor agonist NECA or the adenylate cyclase activator forskolin (both n=3).

The results demonstrate a selective effect of AH 6809 on PGE_2-induced suppression of human neutrophil activation, an effect that cannot be attributed to an action on DP receptors. The EP receptor on human neutrophils, like those mediating relaxation of the cat trachea and chick ileum, is classified as EP_2 on the basis of agonist potency order. However its sensitivity to AH 6809 appears to distinguish it from those receptors. While it is tempting to suggest that these data provide evidence for a receptor subtype, the lack of detailed information in the literature on the action of AH 6809 in EP_2 receptor containing tissues means that experimental differences cannot be excluded.

AH 6809, SC 19220 and BW A868C were gifts from Glaxo, Searle and Wellcome respectively.

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HUMAN NEUTROPHIL & MONOCYTE RESPONSES TO PGE_2 ARE INHIBITED BY THE PROSTANOID ANTAGONIST AH 6809

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Prostaglandin E (EP) receptor agonist potency order suggests that human neutrophils and monocytes have EP_2 receptors. However, there are no selective antagonists for the EP_2 receptor. The activity of the EP1/DP receptor antagonist AH 6809 on responses to PGE_2 in human neutrophils and monocytes has therefore been evaluated. The inhibition by PGE_2 (pEC_{50}=6.9) of fMLP (0.1μM) stimulated superoxide generation by isolated neutrophils was antagonised by AH 6809 (pA_2=7.0, slope=1.1±0.1) but not by antagonists for EP_1 (SC 19220) or DP (BW A868C) receptors. AH 6809 (10μM) did not affect inhibition of neutrophil superoxide generation by the adenosine receptor agonist NECA or by forskolin. In addition, elevation of cAMP levels by PGE_2 in both isolated neutrophils (pEC_{50}=6.8) and monocytes (pEC_{50}=6.5) were also antagonised by AH 6809 (DR=1.3 & 1.6 log units, respectively). Whether this sensitivity to AH 6809 on neutrophils and monocytes, compared with other EP_2 tissues, is due to experimental differences or receptor divergence is unclear.

Comparison of prostanoid DP-receptors in the rabbit isolated saphenous vein and human neutrophil.

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Prostanoid receptors have been designated DP, EP, FP, IP and TP-receptors on the basis of agonist and antagonist data and furthermore, EP-receptors have been subclassified into four distinct subtypes (Coleman *et al.*, 1994). It has also been postulated that there may be subtypes of the DP-receptor (Woodward *et al.*, 1993). In the present study we have examined the effects of selective DP-receptor ligands in the rabbit saphenous vein (RbSV) and human neutrophil (PMN).

Isolated rings of saphenous vein from male NZW rabbits were prepared as described previously (Lydford *et al.*, 1994) with 10μM GR32191B in the buffer. Human PMNs were prepared according to Li *et al.*, 1993, for measurement of fMLP stimulated superoxide production. In both preparations, concentration-effect (E/[A]) curves to DP-receptor agonists were constructed in the absence and presence of appropriate antagonists. Data are presented as mean ± s.e ($n \geq 3$).

In the RbSV, the DP-receptor agonists BW245C, ZK 118.182 and PGD_2 caused concentration-dependent relaxations with mean potencies ($p[A_{50}]$) of 7.58 ± 0.11, 7.36 ± 0.05 and 7.00 ± 0.07 respectively.
The potent DP-receptor antagonist BW A868C (0.01 - 100 μM), caused a rightward displacement of BW245C E/[A] curves in a manner consistent with a two receptor system (Lemoine and Kaumann, 1983). Analysis of the data using their model yielded two pK_B estimates of *8.50 ± 0.07 and 4.89 ± 0.08. The higher estimate corresponds to an activity at DP-receptors, the latter to EP_4-receptors (Lydford *et al.*, 1994). The putative DP-receptor antagonist ZK 138.357 (Thierauch *et al.*, 1995) was without significant effect on BW245C E/[A] curves at a concentration of 1 μM however at 30μM, a three-fold shift of BW245C E/[A] curves was observed. In addition, E/[A] curves to BW245C were antagonised by the EP_1/DP-receptor antagonist AH6809 (3 - 30 μM).

In human PMNs, the $p[A_{50}]$s for inhibition of superoxide production by BW245C, ZK 118.182 and PGD_2 were 8.69 ± 0.10, 7.92 ± 0.12 and 7.73 ± 0.14 respectively.
BW A868C (0.1 μM), ZK 138.357 (1 μM) and AH6809 (3 - 30 μM) inhibited these responses with antagonist potencies similar to previously published estimates for DP-receptor blockade (Table 1).

Table 1. Antagonist potencies against BW245C (pA_2 or $*pK_B$)

Antagonist:	BW A868C	ZK 138.357	AH6809
RbSV	*8.50 ± 0.07	†5.05 ± 0.12	5.93 ± 0.05
PMN	†9.46 ± 0.27	†7.25 ± 0.03	6.59 ± 0.19

† Estimates obtained from a single concentration of antagonist

Whilst the agonist potency order is the same in both preparations; BW245C > ZK 118.182 > PGD_2; data obtained with a range of DP-receptor antagonists appears to indicate differences between the DP-receptors on the RbSV and human PMN. Possible interpretations of the antagonist data is that the differences are due to species variation or interactions with other prostanoid receptors. However, the change in antagonist potency orders between the two preparations may indicate activities at different receptor subtypes.

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